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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12N 15/86, A61K 48/00	A1	(11) International Publication Number: WO 94/29471 (43) International Publication Date: 22 December 1994 (22.12.94)
(21) International Application Number: PCT/US94/04075 (22) International Filing Date: 13 April 1994 (13.04.94) (30) Priority Data: 08/074,920 10 June 1993 (10.06.93) US 08/218,335 25 March 1994 (25.03.94) US (71) Applicant: GENETIC THERAPY, INC. [US/US]; 938 Clopper Road, Gaithersburg, MD 20878 (US). (72) Inventors: CONNELLY, Sheila; Apartment 21, 783 Quince Orchard Boulevard, Gaithersburg, MD 20878 (US). KALEKO, Michael; 8 Hearthstone Court, Rockville, MD 20854 (US). SMITH, Theodore; 20165 Club Hill Drive, Germantown, MD 20878 (US). (74) Agents: OLSTEIN, Elliot, M. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report.
(54) Title: ADENOVIRAL VECTORS FOR TREATMENT OF HEMOPHILIA (57) Abstract  An adenoviral vector including at least one DNA sequence encoding a clotting factor, such as, for example, Factor VIII, or Factor IX. Such vectors may be administered to a host in an amount effective to treat hemophilia in the host. The vectors infect hepatocytes very efficiently, whereby the hepatocytes express the DNA sequence encoding the clotting factor.		

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### ADENOVIRAL VECTORS FOR TREATMENT OF HEMOPHILIA

This application is a continuation-in-part of application Serial No. 074,920, filed June 10, 1993.

#### Field of the Invention

This invention relates to adenoviral vectors. More particularly, this invention relates to adenoviral vectors which may be employed in the treatment of hemophilia.

#### Background of the Invention

Hemophilias A and B are X-linked, recessive bleeding disorders caused by deficiencies of clotting Factors VIII and IX, respectively. In the United States there are approximately 17,000 patients with hemophilia A and 2,800 with hemophilia B. The clinical presentations for both hemophilias are characterized by episodes of spontaneous and prolonged bleeding. Patients frequently suffer joint bleeds which lead to a disabling arthropathy. Current treatment is directed at stopping the bleeding episodes with intravenous infusions of plasma-derived clotting factors or, for hemophilia A, recombinant Factor VIII. However, therapy is limited by the availability of clotting factors, their short half-lives *in vivo*, and the high cost of treatment, which can approach 100,000 dollars per year.

Gene therapy offers the promise of a new method of treating hemophilia. Several groups of researchers have conducted

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research with retroviral vectors containing RNA encoding Factor VIII and Factor IX. Virtually every attempt to date to produce therapeutic levels of these factors in vivo with such vectors, however, has been unsuccessful. The cDNA and the RNA for Factor VIII has been particularly difficult to work with.

Hoeben, et al., J. Biol. Chem, Vol. 265, pgs 7318-7323 (1990) and Israel, et al. Blood, Vol. 75, No. 5, pgs. 1074-1080 (March 1, 1990) describe the infection of mouse fibroblasts in vitro with retroviral vectors including DNA (RNA) encoding B-domain deleted human Factor VIII. Although such infected cells were found to express functional human Factor VIII in vitro, the protein was expressed at low levels.

Recently, Hoeben, et al., Human Gene Therapy, Vol. 4, pgs 179-186 (1993) infected fibroblasts with retroviral vectors including DNA encoding human Factor VIII. These cells then were implanted into immune-deficient mice. Although cells recovered from the implants up to 2 months post-implantation still had the capacity to secrete Factor VIII when regrown in tissue culture, human Factor VIII was not detected in plasma samples of the recipient mice.

Lynch, et al., Human Gene Therapy, Vol. 4, pgs. 259-272 (1993), describes the transfection of PE501 packaging cells with the plasmid forms of retroviral vectors including human Factor VIII cDNA. The virus was harvested, and used to infect PA317 amphotropic retrovirus packaging cells. The infected cells, however, produced human Factor VIII and virus titer in an amount which was about two orders of magnitude lower than those from similar retroviral vectors containing other cDNAs. Lynch, et al. also observed a 100-fold lower accumulation of vector RNAs containing the human Factor VIII sequences in comparison to vectors containing other cDNA sequences.

Lynch, et al. also reported the following difficulties in working with Factor VIII. High titer human Factor VIII-containing retroviral vector stocks are difficult to generate, and retroviral vectors containing Factor VIII cDNA sequences tend

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to rearrange and/or delete portions of the Factor VIII cDNA sequences. In addition, Factor VIII mRNA is inherently unstable. Also, the B-domain deleted Factor-VIII coding region contains a 1.2 kb RNA accumulation inhibitory signal.

Thus, the literature discloses that there have been significant problems in working with retroviral approaches to gene therapy with Factor VIII and that only limited expression has been achieved. The applicants are not aware of any published reports in which human Factor VIII has been expressed in an animal model.

Researchers also have experienced significant difficulties in attempting to achieve therapeutic levels of Factor IX expression with retroviral vectors.

Palmer, et al., Blood, Vol. 73, No. 2, pgs. 438-445 (February 1989) discloses the transduction of human skin fibroblasts with retroviral vectors including DNA (RNA) encoding human Factor IX. Such transformed fibroblasts then were given to rats and to nude mice. Although such fibroblasts were found to transiently express human Factor IX in the animal blood in amounts up to 190 ng/ml, this amount is not generally considered to be at a therapeutic level.

Scharfmann, et al., Proc. Nat. Acad. Sci., Vol. 88, pgs. 4626-4630 (June 1991) discloses the transduction of mouse fibroblast implants with a retroviral vector including a B-galactosidase gene under the control of the dihydrofolate reductase (DHFR) promoter. Such fibroblasts then were grafted into mice, and expression of the  $\beta$ -galactosidase gene for up to sixty days was obtained. Scharfmann, et al. also disclose fibroblasts transduced with canine Factor IX, but they only obtained short-term and non-therapeutic levels of expression.

Dai, et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 10892-10895 (November 1992) discloses the transfection of mouse primary myoblasts with retroviral vectors including canine Factor IX DNA under the control of a mouse muscle creatine kinase enhancer and a human cytomegalovirus promoter. The transfected myoblasts then

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were injected into the hind legs of mice. Expression of canine Factor IX over a period of 6 months was obtained; however, the steady-state levels of Factor IX secreted into the plasma (10 ng/ml for  $10^7$  injected cells) are not sufficient to be of therapeutic value.

Gerrard, et al., Nature Genetics, Vol. 3, pgs. 180-183 (February 1993), discloses the transfection of primary human keratinocytes with a retroviral vector including a human Factor IX gene under the control of the retroviral LTR. The transformed keratinocytes then were transplanted into nude mice, and human Factor IX was detected in the bloodstream for about 1 week. The amounts of Factor IX, however, were about 2.5 ng/ml, or about 1% of a therapeutic dose.

Kay, et al., Science, Vol. 262, pgs. 117-119 (October 1, 1993) discloses the direct infusion of retroviral vectors including Factor IX DNA into the portal vasculature of dogs following partial hepatectomy. The animals expressed low levels of canine Factor IX for more than 5 months. Although such expression of Factor IX resulted in reductions of whole blood clotting and partial thromboplastin times of the treated animals, the authors stated that increased levels of Factor IX must first be achieved before the technique could be applied to humans.

Zhou, et al., Science in China, Vol. 36, No. 9, pgs. 33-41 (September 1993) discloses the transfection of rabbit skin fibroblasts with retroviral vectors including DNA encoding human Factor IX. The fibroblasts then were implanted into rabbits as autografts or allografts. Expression of the human Factor IX was maintained in the rabbits for over 10 months. Factor IX levels in the rabbit plasma of up to 480 ng/ml were claimed to have been achieved; however, the assay used to measure Factor IX employed an anti-rabbit antibody that had the potential of generating false positive results.

Lu, et al., Science in China, Vol. 36, No. 11, pgs. 1341-1351 (November 1993) and Hsueh, et al., Human Gene Therapy, Vol. 3, pgs. 543-552 (1992) discloses a human gene therapy trial in

which human skin fibroblasts were taken from two hemophiliac patients, and transfected with retroviral vectors including DNA encoding human Factor IX. The cells then were pooled and embedded in a collagen mixture, and the cells then were injected into the patients. In one patient, the concentration of human Factor IX increased from 71 ng/ml to 220 ng/ml, with a maximum level of 245 ng/ml. The clotting activity of this patient increased from 2.9% to 6.3% of normal. In the other patient, the plasma level of Factor IX increased from 130 ng/ml to 250 ng/ml, and was maintained at a level of 220 ng/ml for 5½ months; however, the clotting activity has not increased. Lack of pretreatment Factor IX data on these patients makes it difficult to interpret the small increases in Factor IX seen in treatment.

The conclusion to be drawn from scientific literature on the attempts to use retroviruses in gene therapy for hemophilia A and hemophilia B is that, in spite of a very concerted effort and numerous attempts, by and large the field has failed to produce retroviral vectors that can be used to achieve therapeutic levels of expression of human Factor VIII or human Factor IX in vivo. Working with Factor VIII has been especially difficult, and the results have been uniformly unsatisfactory. The experimental strategies described above are laborious and clinically invasive.

Adenoviral vectors offer another approach to gene therapy. Adenovirus genomes are linear, double-stranded DNA molecules of approximately 36 kilobase pairs. Each extremity of the viral genome has a short sequence known as the inverted terminal repeat (or ITR), which is necessary for viral replication. The well-characterized molecular genetics of adenovirus render it an advantageous vector for gene transfer. Portions of the viral genome can be substituted with DNA of foreign origin. In addition, recombinant adenoviruses are structurally stable and no rearranged viruses are observed after extensive amplification.

Recombinant adenoviruses have been used as efficient vectors for gene transfer into a number of cell types. There are several reports of hepatocyte transduction: Jaffe, et al., Nature

Genetics, Vol. 1, pgs. 372-378 (1992) (alpha-1-antitrypsin); Li, et al., Human Gene Therapy, Vol. 4, pgs. 403-409 (1993) (beta-galactosidase); Stratford-Perricaudet, et al., Human Gene Therapy, Vol. 1, pgs. 241-256 (1990) (ornithine transcarbamylase); Smith, et al., Nature Genetics, Vol. 5, pgs. 397-402 (1993) (Factor IX); and J. Am. Med. Assoc., Vol. 269, No. 7, pg. 838 (February 17, 1993) (marker protein).

Because Factor VIII is synthesized largely in hepatocytes (Kelly, et al. Br. J. Haemat., Vol. 56, pgs. 535-543 (1984); Wion, et al., Nature, Vol. 317, pgs. 726-729 (1985); Zelechovska, et al. Nature, Vol. 317, pgs. 729-732 (1985)), transduction of hepatocytes with a Factor VIII - containing recombinant adenovirus, resulting in the expression of Factor VIII protein *in vivo*, may be an effective gene therapy-based treatment for hemophilia A.

The inventors have discovered how to produce high titer, stable, adenoviral vectors that produce therapeutic levels of clotting factors when administered to an animal host. These vectors mediate gene transfer *in vivo* and will enable treatment protocols to be much less laborious and invasive than those previously described.

#### Brief Description of the Drawings

The invention will now be described with respect to the drawings, wherein:

Figure 1 is a schematic of the construction of plasmid pG1.

Figure 2 is the sequence of the multiple cloning site in the pG1 plasmid;

Figure 3 is a map of plasmid pG1;

Figure 4 is a map of plasmid pG1H9;

Figure 5 is a map of plasmid phfacIX;

Figure 6 is a map of plasmid pG1H9B;

Figure 7 is a schematic of the construction of plasmid pHR;



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Figure 8 is a schematic of the assembly of an ITR, encapsidation signal, Rous Sarcoma Virus promoter, adenoviral tripartite leader sequence, and a linking sequence using PCR amplification;

Figure 9 is a schematic of the construction of pAvS6;

Figure 10 is a map of pAvS6;

Figure 11 is the human Factor IX cDNA sequence;

Figure 12 is a map of pAvS6H9B;

Figure 13 is a schematic of the generation of AvlH9B;

Figure 14 is a graph of plasma levels of Factor IX in mice given intraparenchymal or portal vein injections of AvlH9B;

Figure 15 is an autoradiogram of a Southern analysis to determine the presence of Factor IX DNA in mouse liver;

Figure 16 is a map of plasmid pMT2LA;

Figure 17 is the sequence of B-domain deleted human Factor VIII cDNA;

Figure 18 is a map of plasmid pAvS6H81;

Figure 19 is a schematic of the construction of AvlH81;

Figure 20 is a map of plasmid pAT2-3eG.

Figure 21 is a map of plasmid pAvAL1;

Figure 22 is a map of plasmid pGEM(sac);

Figure 23 is a map of plasmid pGEM;

Figure 24 is a map of plasmid pGEMalb;

Figure 25 is a map of plasmid pGEMalbf8B;

Figure 26 is a map of plasmid pAvALH81;

Figure 27 is a map of plasmid pgemF8B2;

Figure 28 is a map of plasmid pBGS19-AIgI;

Figure 29 is a map of plasmid pUC19;

Figure 30 is a map of plasmid pUC19-AIgI;

Figure 31 is a map of plasmid pBGS19;

Figure 32 is a map of plasmid pGemAPF8B;

Figure 33 is a map of plasmid pAvAPH81;

Figure 34 is a map of plasmid pGemAPexF8;

Figure 35 is a map of plasmid pALAPF8B;

Figure 36 is a map of plasmid pAvALAPH81;

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Figure 37 is a schematic of the generation of Av1ALH81;

Figure 38 is a blot of a restriction digestion analysis of Av1ALH81 DNA;

Figure 39 is a schematic of adenoviral vectors Ad5-dl327, Av1ALH81, and Av1ALAPH81;

Figure 40 is a standard log-log curve of a human Factor VIII - specific ELISA assay;

Figures 41 and 42 are graphs of the amounts of human Factor VIII in mouse plasma over time in two separate experiments;

Figure 43 is a graph of in vivo expression of human Factor VIII in mouse plasma over time after injection of various doses of Av1ALH81;

Figures 44 and 45 are graphs of human Factor VIII half-life in experimental mice and control mice;

Figure 46 is a graph of in vivo expression of human Factor VIII in mice given  $4 \times 10^9$  pfu of Av1ALH81;

Figure 47 is a map of plasmid pBLSKH9CI;

Figure 48 is a map of plasmid pBLSKH9D;

Figure 49 is a map of Plasmid pBLH9CINT;

Figure 50 is a map of plasmid pBLH9EINT;

Figure 51 is a map of plasmid pBLH9E;

Figure 52 is a map of plasmid pBLH9F;

Figure 53 is a map of plasmid pAV1H9D;

Figure 54 is a map of plasmid pAV1H9ER;

Figure 55 is a map of plasmid pAV1H9FR;

Figure 56 is a graph of Factor IX expression in mice treated with Av1H9B, Ad1H9D, Ad1H9ER and Ad1H9FR;

Figure 57 is a graph of Factor IX expression in mice treated with  $1 \times 10^9$  pfu of Av1H9B, Ad1H9D, or Ad1H9ER;

Figure 58 is a graph of Factor IX expression in mice treated with  $5 \times 10^7$  pfu of Ad1H9ER or Ad1H9FR; and

Figure 59 is a graph of in vitro expression of Factor IX in HepG2 and HeLa cells, and of in vivo expression of Factor IX in mice treated with  $2 \times 10^8$  pfu of Av1H9B, Ad1H9D, or Ad1H9ER. In

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each group of 3 bars, the leftmost bar represents data for AvlH9B, the middle bar, AdlH9D, and the rightmost bar, AdlH9ER.

#### Detailed Description of the Invention

In accordance with an aspect of the present invention, there is provided an adenoviral vector including at least one DNA sequence encoding a clotting factor.

The term "DNA sequence encoding a clotting factor" as used herein means DNA which encodes a full-length clotting factor or a fragment, derivative, or analogue of a clotting factor, i.e., such DNA may be a full-length gene encoding a full-length clotting factor, or a truncated gene, or a mutated gene encoding a fragment or derivative or analogue of such clotting factor which has clotting factor activity. The term "DNA sequence" refers generally to a polydeoxyribonucleotide molecule and more specifically to a linear series of deoxyribonucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of the adjacent pentoses.

In one embodiment, the DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof having Factor VIII clotting activity. In another embodiment, the DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof having Factor IX clotting activity.

The DNA sequence encoding human Factor IX is shown and described in U.S. Patent No. 4,994,371 issued February 19, 1991 to Davie, et al. and European Patent No. EP 0 107 278 B1 (publication of grant November 15, 1989) to National Research Development Corporation. DNA sequences encoding Factor VIII and fragments or derivatives thereof are shown and described in U.S. Patent Nos. 4,757,006 issued July 12, 1988 to Toole, Jr. et al.; 4,868,112 issued September 19, 1989 to Toole, Jr.; 5,045,455 issued September 3, 1991 to Kuo, et al.; 5,004,804 issued April 2, 1991 to Kuo, et al.; 5,112,950 issued May 12, 1992 to Meulien, et al.; and 5,149,637 issued September 22, 1992 to Scandella, et al.

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The inventors have found that, by infecting host cells in vivo with adenoviral vectors including at least one DNA sequence encoding a clotting factor, one is able to achieve expression, in vivo, of the clotting factor, or fragment or derivative or analogue of such clotting factor having clotting factor activity, at effective therapeutic levels. In general, such effective therapeutic levels are about 5% or greater of the normal level of the clotting factor (N. Engl. J. Med., Vol. 328, No. 7, pgs. 453-459 (February 18, 1993); Blood, Vol. 74, No. 1, pgs. 207-212 (July 1989)). Such levels are, in general, for Factor VIII, about 10 ng/ml or greater, and for Factor IX are about 250 ng/ml or greater.

The DNA sequence encoding a clotting factor is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus promoter; the Rous Sarcoma Virus (RSV) promoter; the albumin promoter; inducible promoters, such as the Mouse Mammary Tumor Virus (MMTV) promoter; the metallothionein promoter; heat shock promoters; the  $\alpha$ -1-antitrypsin promoter; the hepatitis B surface antigen promoter; the transferrin promoter; the apolipoprotein A-1 promoter; the Factor VIII promoter; and the Factor IX promoter. It is to be understood, however, that the scope of the present invention is not to be limited to specific promoters.

In one embodiment, when the DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof, the promoter controlling the DNA sequence is preferably a tissue-specific promoter, such as, for example, the mouse albumin promoter, which is active in liver cells. Although the scope of this embodiment is not intended to be limited to any theoretical reasoning, the inventors believe that, by employing a tissue-specific promoter, possible Factor VIII toxicity to the producer cells is avoided.

When one employs a mouse albumin promoter, which is active

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in liver cells, the adenoviral vectors are preferably grown in cells other than liver cells. When the generated adenoviral vectors are to be administered to a host, such vectors are administered to a host by means known to those skilled in the art, whereby the vectors will travel to and infect liver cells. The infected liver cells then will express Factor VIII in therapeutic amounts. Factor VIII is not toxic to liver cells and thus will continue to be expressed at therapeutic levels.

In yet another embodiment, when the DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof, the promoter controlling the DNA sequence is preferably a strong promoter that is not tissue-specific, such as, for example, the Rous Sarcoma Virus promoter. Because it is believed that Factor IX is not toxic to most cells, the adenoviral vectors may be grown in any cell type, and may be administered to a patient in an effective therapeutic amount, whereby the adenoviral vectors will travel to and infect cells such as liver cells, for example, whereby the Factor IX will be expressed in therapeutic amounts.

Several reports have revealed that, in transgenic mice, enhanced expression of cDNA's can be obtained by the incorporation of 5' and 3' untranslated regions as well as introns (Choo, et al. Nucl. Acids Res., Vol. 15, pgs. 881-884 (1987); Brinster, et al. PNAS, Vol. 85, pgs 836-840 (1988); Jallat, et al. EMBO J., Vol. 9, No. 10, pgs. 3295-3301 (1990); and Choi, et al. Mol. Cell. Biol., Vol. 11, pgs. 3070-3074 (1991)). The effectiveness, however, of genomic elements in improving expression of exogenous genes incorporated into an adenoviral vector backbone has not been demonstrated previously.

In one embodiment, the DNA sequence encoding a clotting factor also may include introns and other genomic elements to enhance expression. The term "genomic element," as used herein, means a sequence of nucleotides in a naturally occurring gene that is not normally incorporated into the cDNA, and which is not part of the adenoviral genome. Such genomic elements which may be included in the vector include, but are not limited to,

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introns, the 5' untranslated region, and the 3' untranslated region of the gene encoding the clotting factor, or portions of such 5' and 3' untranslated regions and introns. Examples of introns which may be employed include, but are not limited to, any of the seven introns of the Factor IX gene, or portions thereof (EMBO J., Vol. 9, No. 10, pgs. 3295-3301 (1990)); or any of the twenty-five introns of the Factor VIII gene (Gitschier, Nature, 312:326-330 (1984)), or portions thereof; or the first exon and intron of the apolipoprotein A-1 gene.

When the DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof, the vector may, in one embodiment, further include the full 3' untranslated region of the Factor IX DNA sequence. In another embodiment, the vector may further include the full 5' untranslated region and a centrally truncated first intron. In yet another embodiment, the vector may further include the full 3' untranslated region, the full 5' untranslated region, and a centrally truncated first intron. Most preferably, the vector contains all of these elements. In a further embodiment, the vector may further include the full 7th intron of the Factor IX gene.

When such elements are included in the vector, improved levels of expression of Factor IX are obtained. Although the scope of the present invention is not intended to be limited to any theoretical reasoning, such improved expression may be due to (i) the incorporation of enhancers in the genomic sequences; (ii) stabilization of the mRNA; (iii) improved processing and transport of the mRNA to the cytoplasm; and/or (iv) improved polyadenylation.

In another embodiment, the first exon and first intron of the apolipoprotein protein A-1 gene may be employed, if desired, with the apolipoprotein A-1 gene promoter. (PNAS, Vol. 80, pgs. 6147-6151 (October 1983); J. Biol. Chem., Vol. 266, No. 27, pgs. 18045-18050 (Sept. 1991)). The above-mentioned introns and/or exons also may be used in combination with the 5' untranslated

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region and/or the 3' untranslated region of the gene encoding the clotting factor.

In one preferred embodiment, the apolipoprotein A-1 promoter may be employed, alone or in combination with the first exon and/or first intron of the apolipoprotein A-1 gene, in combination with the Factor VIII gene.

The adenoviral vector which is employed may, in one embodiment, be an adenoviral vector which includes essentially the complete adenoviral genome (Shenk, et al., Curr. Top. Microbiol. Immunol., 111(3): 1-39 (1984). Alternatively, the adenoviral vector may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted.

In the preferred embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a clotting factor; and a promoter controlling the at least one DNA sequence encoding a clotting factor. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter. In one embodiment, the vector is also free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences.

In another embodiment, the vector is free of at least the majority of the adenoviral E1 and E3 DNA sequences, and is free of one of the E2 and E4 DNA sequences, and is free of a portion of the other of the E2 and E4 DNA sequences.

In still another embodiment, the gene in the E2a region that encodes the 72 kilodalton binding protein is mutated to produce a temperature sensitive protein that is active at 32°C, the temperature at which the viral particles are produced, but is inactive at 37°C, the temperature of the animal or human host. This temperature sensitive mutant is described in Ensinger, et al., J. Virology, 10:328-339 (1972), Van der Vliet, et al., J.

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Virology, 15:348-354 (1975), and Friefeld, et al., Virology, 124:380-389 (1983).

In yet another embodiment, the vector is free of at least the majority of the E1 and E3 DNA sequences, is free of at least a portion of at least one DNA sequence selected from the group consisting of the E2 and E4 DNA sequences, and is free of DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5' end, the "critical left end elements," which include an adenoviral 5' ITR, an adenoviral encapsidation signal, and an Ela enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a multiple cloning site (which may be as hereinabove described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. The vector also may contain a tripartite leader sequence. The DNA segment corresponding to the adenoviral genome serves as a substrate for homologous recombination with a modified or mutated adenovirus, and such sequence may encompass, for example, a segment of the adenovirus 5 genome no longer than from base 3329 to base 6246 of the genome. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAVS6, shown in Figure 10. A desired DNA sequence encoding a clotting factor may then be inserted into the multiple cloning site to produce a plasmid vector.

This construct is then used to produce an adenoviral vector. Homologous recombination is effected with a modified or mutated adenovirus in which at least the majority of the E1 and E3 adenoviral DNA sequences have been deleted. Such homologous recombination may be effected through co-transfection of the plasmid vector and the modified adenovirus into a helper cell



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line, such as 293 cells, by CaPO<sub>4</sub> precipitation. Upon such homologous recombination, a recombinant adenoviral vector is formed that includes DNA sequences derived from the shuttle plasmid between the Not I site and the homologous recombination fragment, and DNA derived from the E1 and E3 deleted adenovirus between the homologous recombination fragment and the 3' ITR.

In one embodiment, the homologous recombination fragment overlaps with nucleotides 3329 to 6246 of the adenovirus 5 (ATCC VR-5) genome.

Through such homologous recombination, a vector is formed which includes an adenoviral 5' ITR, an adenoviral encapsidation signal; an Ela enhancer sequence; a promoter; at least one DNA sequence encoding a clotting factor; a poly A signal; adenoviral DNA free of at least the majority of the E1 and E3 adenoviral DNA sequences; and an adenoviral 3' ITR. The vector also may include a tripartite leader sequence. This vector may then be transfected into a helper cell line, such as the 293 helper cell line, which will include the Ela and Elb DNA sequences, which are necessary for viral replication, and to generate infectious adenoviral particles.

In one embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a clotting factor; and a promoter controlling the at least one DNA sequence encoding a clotting factor. The vector is free of the adenoviral E1, E2, E3, and E4 DNA sequences, and the vector is free of DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter; i.e., the vector is free of DNA encoding adenoviral structural proteins.

Such vectors may be constructed by removing the adenoviral 5' ITR, the adenoviral 3' ITR, and the adenoviral encapsidation signal, from an adenoviral genome by standard techniques. Such components, as well as a promoter (which may be an adenoviral promoter or a non-adenoviral promoter), tripartite leader sequence, poly A signal, and selectable marker, may, by standard

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techniques, be ligated into a base plasmid or "starter" plasmid such as, for example, pBluescript II KS-(Stratagene), to form an appropriate cloning vector. The cloning vector may include a multiple cloning site to facilitate the insertion of the at least one DNA sequence encoding a clotting factor into the cloning vector. In general, the multiple cloning site includes "rare" restriction enzyme sites; i.e., sites which are found in eukaryotic genes at a frequency of from about one in every 10,000 to about one in every 100,000 base pairs. An appropriate vector in accordance with the present invention is thus formed by cutting the cloning vector by standard techniques at appropriate restriction sites in the multiple cloning site, and then ligating the DNA sequence encoding a clotting factor into the cloning vector.

The vector is then packaged into infectious, nonreplicating, recombinant adenoviral particles, using a helper adenovirus which provides the necessary encapsidation materials. Preferably the helper virus has a defective encapsidation signal in order that the helper virus will not encapsidate itself. An example of an encapsidation defective helper virus which may be employed is described in Grable, et al., J. Virol., Vol. 66, pgs. 723-731 (1992).

The vector and the encapsidation defective helper virus are transfected into an appropriate cell line for the generation of infectious viral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes. Examples of appropriate cell lines include, but are not limited to, HeLa cells or 293 (embryonic kidney epithelial) cells (ATCC No. CRL 1573). The infectious viral particles (i.e., the adenoviral vector) may then be transduced into eukaryotic cells, such as hepatocytes, whereby the at least one DNA sequence encoding a clotting factor is expressed by the eukaryotic cells in a host.

The vector, consisting of infectious, but replication-defective, viral particles, which contain at least one DNA

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sequence encoding a clotting factor, is administered in an amount effective to treat hemophilia in a host. In one embodiment, the vector particles may be administered in an amount of from 1 plaque forming unit to about  $10^{14}$  plaque forming units, preferably from about  $1 \times 10^6$  plaque forming units to about  $1 \times 10^{13}$  plaque forming units. The host may be a human or non-human animal host. The preferred non-human animal host is a mammal, most preferably a dog or a non-human primate.

Preferably, the infectious vector particles are administered systemically, such as, for example, by intravenous administration (such as, for example, via peripheral vein injection) or administered via the portal vein, to the bile duct, intramuscularly, intraperitoneally, or intranasally.

The vector particles may be administered in combination with a pharmaceutically acceptable carrier suitable for administration to a patient. The carrier may be a liquid carrier (for example, a saline solution), or a solid carrier, such as, for example, microcarrier beads.

As hereinabove stated, the inventors have found that the incorporation of genomic elements into the adenoviral vector provides for enhanced expression of the DNA sequence encoding a clotting factor. Thus, in accordance with another aspect of the present invention, there is provided an adenoviral vector including at least one DNA sequence encoding a heterologous protein, and at least one genomic element affecting the expression of such DNA sequence. The term "genomic element" is used as previously defined. Such genomic elements include, but are not limited to, introns, the 5' untranslated region, and the 3' untranslated region, and portions of said introns and 3' and 5' untranslated regions. The adenoviral vector may be as hereinabove described.

The DNA sequence encoding a heterologous protein may be a DNA sequence which encodes at least one therapeutic agent. The term "therapeutic" is used in a generic sense and includes treating agents, prophylactic agents, and replacement agents.

DNA sequences encoding therapeutic agents which may be placed into the adenoviral vector include, but are not limited to, DNA encoding Factor VIII and Factor IX as hereinabove described; DNA encoding cytokines; DNA sequences encoding tumor necrosis factor (TNF) genes, such as TNF- $\alpha$ ; genes encoding interferons such as Interferon- $\alpha$ , Interferon- $\beta$ , and Interferon- $\gamma$ ; genes encoding interleukins such as IL-1, IL-1 $\beta$ , and Interleukins 2 through 14; genes encoding GM-CSF; genes encoding adenosine deaminase, or ADA; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; genes encoding soluble CD4; T-cell receptors; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin ( $\alpha$ 1AT) gene, the ornithine transcarbamylase (OTC) gene, the CFTR gene, the insulin gene, viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, and antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A non-B virus.

Promoters which control the DNA sequence may be selected from those hereinabove described.

In one embodiment, the genomic element and the DNA sequence encoding a heterologous protein are part of the same endogenous gene. For example, the adenoviral vector may include DNA encoding Factor IX and a Factor IX genomic element(s). In another embodiment, the DNA sequence encoding a heterologous protein and the genomic element are taken from different endogenous genes. For example, the adenoviral vector may include DNA encoding Factor VIII and Factor IX genomic elements.

In yet another embodiment, an adenoviral vector may be constructed wherein the adenoviral vector includes DNA encoding a heterologous protein and at least one genomic element(s) from the same endogenous gene. The DNA encoding a heterologous protein

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may be modified such that at least one exon is normally present in the DNA encoding the heterologous protein is removed and replaced with one or more exons present in another gene.

Although the scope of this aspect of the present invention is not to be limited to any theoretical reasoning, Applicants believe that, by the inclusion of at least one genomic element in an adenoviral vector including at least one DNA sequence encoding a heterologous protein, one is able to approximate endogenous transcription, RNA processing, and translation of the DNA sequence encoding a heterologous protein, thereby providing for increased expression of the heterologous protein.

The invention will now be described with respect to the following examples; however, the scope of the present invention is not to be limited thereby.

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Example 1Construction of an Adenoviral Vector Including a Factor IX GeneA. Construction of pG1H9

Plasmid pG1 (described in PCT application No. WO91/10728, published July 25, 1991) (Figure 3) was constructed from pLNSX, Miller, et al., Biotechniques, 7:980-990 (1989). The construction strategy for plasmid pG1 is shown in Figure 1. The 1.6 kb EcoRI fragment, containing the 5' Moloney Sarcoma Virus (MoMuSV) LTR, and the 3.0 kb EcoRI/ClaI fragment, containing the 3' LTR, the bacterial origin of replication and the ampicillin resistance gene, were isolated separately. A linker containing seven unique cloning sites was then used to close the EcoRI/ClaI fragment on itself, thus generating the plasmid pGO. The plasmid pGO was used to generate the vector plasmid pG1 by the insertion of the 1.6 kb EcoRI fragment containing the 5' LTR into the unique EcoRI site of pGO. Thus, pG1 consists of a retroviral vector backbone composed of a 5' portion derived from MoMuSV, a short portion of gag in which the authentic ATG start codon has been mutated to TAG (Bender et al., J. Virol., Vol. 61, pgs. 1639-1649 (1987)), a 54 base pair multiple cloning site (MCS) containing from 5' to 3' the sites EcoRI, NotI, SnaBI, SalI, BamHI, XhoI, HindIII, ApaI, and ClaI, and a 3' portion of MoMuLV from base pairs 7764 to 7813 numbered as described in (Van Beveren et al., Cold Spring Harbor, Vol. 2, pg. 567, 1985). (Figure 2). The MCS was designed to generate a maximum number of unique insertion sites, based on a screen of non-cutting restriction enzymes of the pG1 plasmid, the neo<sup>R</sup> gene, the  $\beta$ -galactosidase gene, and hygromycin<sup>R</sup> gene, and the SV40 promoter.

pG1 (Figure 3) was cut with BamHI and HindIII. pLIXSN (Palmer, et al, Blood, Vol. 73, No. 2, pgs. 438-445 (February 1989)), which contains a Factor IX gene, an SV40 promoter, and a neo<sup>R</sup>gene, was also cut with BamHI and HindIII. The resulting

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BamHI-HindIII fragment, which contains the Factor IX gene, was then ligated to the BamHI-HindIII digested pG1 to form pG1H9. (Figure 4). The Factor IX gene could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,994,371.

#### B. Construction of pG1H9B

pG1H9B (Figure 6) was constructed so that the 5' portion of the human Factor IX cDNA starting at the first ATG is identical to the natural 5' human Factor IX sequence. Such is not the case for pG1H9 because of an inversion in the DNA sequence.

pG1H9B was constructed as follows. First, a cDNA clone of human Factor IX was generated by PCR amplification of human liver cDNA followed by subcloning into the plasmid pBluescript SK- (Stratagene, La Jolla, California). The resulting plasmid was designated phfacIX (Figure 5). The 5' end of the Factor IX sequence in this plasmid was then used to replace the 5' end of the Factor IX sequence in G1H9. phfacIX then was cut with BamHI and DraI, and the 334 bp fragment corresponding to the 5' end of the Factor IX cDNA was isolated. pG1H9 was cut with DraI and ClaI and the 1253 bp fragment encoding the 3' end of the Factor IX cDNA was isolated. The two isolated DNA fragments encoding Factor IX cDNA were ligated into the pG1H9 backbone which had been cut with BamHI and ClaI to generate pG1H9B (Figure 6).

#### C. Construction of pAVS6

The adenoviral construction shuttle plasmid pAVS6 was constructed in several steps using standard cloning techniques including polymerase chain reaction based cloning techniques. First, the 2913 bp BglII, HindIII fragment was removed from Ad-dl327 and inserted as a blunt fragment into the XhoI site of pBluescript II KS-(Stratagene, La Jolla, CA) (Figure 7).

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Ad-dl327 is identical to adenovirus 5 except that an XbaI fragment including bases 28591 to 30474 (or map units 78.5 to 84.7) of the Adenovirus 5 genome, and which is located in the E3 region, has been deleted. The E3 deletion in Ad-dl327 is similar to the E3 deletion in Ad-dl324, which is described in Thimmapaya, et al., Cell, Vol. 31, pg. 543 (1983). The complete Adenovirus 5 genome is registered as Genbank accession #M73260, incorporated herein by reference, and the virus is available from the American Type Culture Collection, Rockville, Maryland, U.S.A. under accession number VR-5.

Ad-dl327 was constructed by routine methods from Adenovirus 5 (Ad5). The method is outlined briefly as follows and previously described by Jones and Shenk, Cell 13:181-188, (1978). Ad5 DNA is isolated by proteolytic digestion of the virion and partially cleaved with Xba 1 restriction endonuclease. The Xba 1 fragments are then reassembled by ligation as a mixture of fragments. This results in some ligated genomes with a sequence similar to Ad5, except excluding sequences 28591 bp to 30474 bp. This DNA is then transfected into suitable cells (e.g. KB cells, HeLa cells, 293 cells) and overlaid with soft agar to allow plaque formation. Individual plaques are then isolated, amplified, and screened for the absence of the 1878 bp E3 region Xba 1 fragment.

The orientation of this fragment was such that the BglII site was nearest the T7 RNA polymerase site of pBluescript II KS<sup>-</sup> and the HindIII site was nearest the T3 RNA polymerase site of pBluescript II KS<sup>-</sup>. This plasmid was designated pHR. (Figure 7).

Second, the ITR, encapsidation signal, Rous Sarcoma Virus promoter, the adenoviral tripartite leader (TPL) sequence and linking sequences were assembled as a block using PCR amplification (Figure 8). The ITR and encapsidation signal (sequences 1-392 of Ad-dl327 [identical to sequences from Ad5, Genbank accession #M73260] incorporated herein by reference) were amplified (amplification 1) together from Ad-dl327 using primers



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containing NotI or AscI restriction sites. The Rous Sarcoma Virus LTR promoter was amplified (amplification 2) from the plasmid pRC/RSV (sequences 209 to 605; Invitrogen, San Diego, CA) using primers containing an AscI site and an SfiI site. DNA products from amplifications 1 and 2 were joined using the "overlap" PCR method (amplification 3) (Horton, et al., BioTechniques, 8:528-535 (1990)) with only the NotI primer and the SfiI primer. Complementarity between the AscI containing end of each initial DNA amplification product from reactions 1 and 2 allowed joining of these two pieces during amplification. Next the TPL was amplified (amplification 4) (sequences 6049 to 9730 of Ad-dl327 [identical to similar sequences from Ad5, Genbank accession #M73260]) from cDNA made from mRNA isolated from 293 cells (ATCC Accession No. CRL 1573) infected for 16 hrs. with Ad-dl327 using primers containing SfiI and XbaI sites respectively. DNA fragments from amplification reactions 3 and 4 were then joined using PCR (amplification 5) with the NotI and XbaI primers, thus creating the complete gene block.

Third, the ITR-encapsidation signal-TPL fragment was then purified, cleaved with NotI and XbaI and inserted into the NotI, XbaI cleaved pHR plasmid. This plasmid was designated pAvS6A and the orientation was such that the NotI site of the fragment was next to the T7 RNA polymerase site (Figure 9).

Fourth, the SV40 early polyA signal was removed from SV40 DNA as an HpaI-BamHI fragment, treated with T4 DNA polymerase and inserted into the SalI site of the plasmid pAvS6A-(Figure 9) to create pAvS6 (Figures 9 and 10).

#### D. Construction of Av1H9B

Factor IX cDNA (Figure 11), which contains the entire protein coding sequence, 26 base pairs of 5' untranslated DNA (assuming translation starts at the third ATG of the message) and 160 base pairs of 3' untranslated DNA, was excised from pG1H9B by restriction digestion with ClaI, followed by filling in the 5'

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overhang using Klenow, followed by restriction digestion with SmaI. The Factor IX cDNA could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,994,371.

The fragment encoding Factor IX was isolated by electrophoresis in a 1.0% agarose gel followed by electroelution of the DNA. This fragment was subcloned into pAvS6 which had been linearized with EcoRV and treated with calf intestinal phosphatase. The resulting shuttle plasmid pAvS6H9B (Figure 12), contains the 5' inverted terminal repeat of adenovirus type 5 (Ad 5), the origin of replication of Ad 5, the Ad 5 encapsidation signal, the Ela enhancer, the RSV promoter, the tripartite leader sequence of Ad 5, Factor IX cDNA, the SV40 early polyadenylation signal, and Ad 5 sequences from nucleotide positions 3329-6246.

The recombinant adenoviral vector Av1H9B was generated as depicted in Figure 13.  $1.5 \times 10^6$  293 cells were cotransfected in a 60 mm tissue culture dish with  $4\mu\text{g}$  of the large Cla I fragment of Ad-d1327 (an E3 deletion mutant of Ad 5) and  $5\mu\text{g}$  of shuttle plasmid pAvS6H9B digested with Not I and Kpn I. Transfections were done using BRL's Transfinity calcium phosphate transfection system. Approximately 15 hours after transfection, medium containing DNA/calcium phosphate precipitate was removed from the dishes, the cells were gently washed with PBS, then overlaid with a 1:1 mixture of 2x MEM (GIBCO's 2x Modified Eagle Medium supplemented with 15% FBS) and 2% SeaPlaque agarose.

Plaques were picked using sterile Pasteur pipettes and transferred into 0.1 ml of infection medium (Improved Minimum Essential Medium (IMEM), 1% FBS) in an Eppendorf tube. Resuspended plaques were subjected to three freeze/thaw cycles, then cleared of cell debris by a 15 second centrifugation at full speed in a microfuge.

Recombinant adenovirus was amplified in 293 cells as follows. Approximately  $5 \times 10^5$  293 cells per dish were seeded into 30 mm dishes. The next day medium was removed from the cells and replaced with 0.2 ml of infection medium and 0.1 ml of a resuspended plaque. The plates were incubated with gentle

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rocking for 90 minutes in a 37°C, 5% CO<sub>2</sub> incubator. Subsequently, 2 ml of complete medium (IMEM, 10% FBS) were added. Approximately 40 hours later a cytopathic effect was clearly visible; cells were rounded-up and beginning to detach from the plate. Cells and medium were transferred to plastic tubes, subjected to four freeze/thaw cycles, and centrifuged at 2000 x g for 5 minutes. The resulting supernatant is referred to as the crude viral lysate (CVL1).

Viral DNA was isolated from an aliquot of each CVL1, then analyzed for the presence of Factor IX cDNA by PCR, as follows. A 60 µl aliquot of supernatant was transferred into an Eppendorf tube and incubated at 80°C for 5 minutes. The sample was centrifuged at full speed for 5 minutes in a microfuge, then 5 µl of the supernatant were used for PCR analysis. PCR analysis was done using the Perkin Elmer Cetus GeneAmp kit. Two different pairs of primers which amplify different portions of the human Factor IX cDNA were used. All samples yielded the expected amplified band.

#### Example 2

##### In vitro and in vivo function of the vector of Example 1

Recombinant adenovirus vectors containing Factor IX cDNA were tested for their ability to express human Factor IX in 293 cells. Approximately 5 x 10<sup>5</sup> 293 cells were seeded per 60 mm dish. The next day, medium was replaced with 0.1 ml of recombinant adenovirus and 0.1 ml of infection medium. Plates were incubated for 1 hour with gentle rocking at 37°C in 5% CO<sub>2</sub>, followed by addition of 4 ml of complete medium. The cells were gently washed five times with PBS, then 4 ml of complete medium were added. Media samples were collected 24 hours later and centrifuged at 1500 x g for 5 minutes. Supernatants were assayed for human Factor IX by ELISA (Asserachrom IX:Ag ELISA kit, American Bioproducts), and the levels were 445 and 538 ng/ml for

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the two samples, demonstrating that the recombinant adenoviral vectors are able to express human Factor IX. Uninfected 293 cells yielded background levels of Factor IX.

One recombinant adenovirus was selected for a large scale virus preparation. Approximately  $5 \times 10^6$  293 cells were seeded onto a 15 cm tissue culture dish. The next day, the medium was replaced with 4 ml of infection medium plus 1 ml of the crude viral stock. Then the plates were incubated at 37°C, 5% CO<sub>2</sub> with gentle rocking for 90 minutes, followed by addition of 15 ml of complete medium. Approximately 40 hours later, when a cytopathic effect was clearly visible, cells and medium were transferred to a 50 ml plastic tube. Cells were lysed by five freeze/thaw cycles and cell debris was removed by centrifugation at 1500 x g for five minutes. This supernatant was termed CVL2.

15 ml of CVL2 then was mixed with 35 ml of infection medium and 5 ml of this mixture was added to each of ten 15 cm plates of nearly confluent 293 cells. The plates were incubated at 37°C, 5% CO<sub>2</sub> with gentle rocking for 1 hour, followed by addition of 15 ml complete medium to each plate. Twenty-four hours later a cytopathic effect was observed; cells were rounded-up, but not lysed. Cells and medium were centrifuged at 2000 x g for 10 minutes. The cell pellet was resuspended in 6 ml of complete medium. Cells were lysed by five freeze/thaw cycles, followed by centrifugation in a SW40 rotor at 7000 rpm for 10 minutes at 4°C. Virus in the supernatant was purified on a CsCl step gradient as follows. 3.0 ml of 1.25 g/ml CsCl in TD buffer (25 mM Tris, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, pH7.5) was placed in an ultraclear Beckman #344060 ultracentrifuge tube. This was underlaid with 3.0 ml of 1.40 g/ml CsCl in TD buffer. The CsCl layers were overlaid with 4.5 ml of viral supernatant. Centrifugation was done at 35,000 rpm, 22°C for 1 hour in a SW40 rotor. Two bands were visible, an upper band that consists of empty capsids and a lower band consisting of intact recombinant adenovirus.

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The lower band was collected with a 3 ml syringe and a 21 gauge needle, and then rebanded as follows. 9.0 ml of 1.33 g/ml CsCl in TD buffer was placed into an ultracentrifuge tube. This was overlaid with the virus collected from the first spin. Centrifugation was done at 35,000 rpm, 22°C for 18 hours. The opalescent band was collected as above and glycerol was added to a final concentration of 10%. The adenovirus was dialyzed against one liter of 10 mM Tris pH 7.4, 10 mM MgCl<sub>2</sub>, and 10% glycerol at 4°C. Dialysis was done for 4 hours and the buffer was changed three times at one hour intervals. The virus was recovered and stored at -70°C in aliquots in sterile Eppendorf tubes. The titer of this virus preparation was  $9.6 \times 10^9$  pfu/ml.

In the first in vivo experiment, the recombinant adenovirus Av1H9B was injected into three C57BL/6 mice by three different methods: an intraparenchymal injection into the liver, infusion into the portal vein, and infusion into the tail vein.

The animal which received an intraparenchymal injection was anesthetized under Metofane. A longitudinal incision approximately 7 mm in length was made just below the xiphoid. Pressure was applied to the flanks causing protrusion of the median and left lateral lobes. For injection, 0.1 ml of virus ( $1 \times 10^9$  pfu) was diluted to 1.0 ml with Hanks Balanced Salt Solution (HBSS). The virus was injected into 4 different sites of the liver: 0.25 ml was injected into each half of the median lobe and into the left and right sides of the left lateral lobe. Each injection was done over approximately one minute. Upon removal of the needle, hemostasis was achieved by placing small pieces of gelfoam over the injection site. After 2 minutes, the gelfoam was removed, the liver was gently placed into the abdominal cavity, and the skin incision was closed with autoclips. Animals awakened within several minutes of surgery and were ambulatory within one hour.

The animal which received a portal vein infusion of AdH9B was anesthetized under Metofane. A midline longitudinal incision was made from the xiphoid to just above the pelvis. The

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intestines were gently externalized to the left side of the animal with wet cotton tip applicators. An 0.1 ml aliquot of virus ( $1 \times 10^9$  pfu) was diluted to 1.0 ml with HBSS. The viral suspension was infused over 30 seconds into the portal vein using a 1 ml syringe and a 27 gauge needle. A 3 x 3 mm piece of gelfoam was placed over the injection site. The needle then was withdrawn. Hemostasis was achieved by applying mild pressure to the gelfoam for 5 minutes using a wet cotton tip applicator. The gelfoam was left in place. The intestines were gently returned to the abdominal cavity. The incision was closed using autoclips. The animal awakened within 30 minutes of surgery and was ambulatory within 1 hour.

A tail vein infusion of Av1H9B was performed using 0.1 ml of virus ( $1 \times 10^9$  pfu) diluted to 1.0 ml with HBSS. The viral suspension was infused over a ten second period using a 27 gauge needle.

The animals which received an intraparenchymal injection and portal vein infusion, as well as a control mouse which received no virus, were bled via the retro-orbital plexus on days 2, 6, and 9 after virus delivery. The animal which received a tail vein infusion was bled 2 days after virus delivery. Plasma levels of human Factor IX were determined by ELISA. The results are shown in Figure 14.

At this point, it was important to determine vector levels in the livers of the mice. Therefore, the animals which received an intraparenchymal injection and a portal vein infusion and the negative control mouse were sacrificed on day 9 after infusion and the mouse which received a tail vein injection was sacrificed on day 2 after infusion. The liver of each mouse was removed and extensively minced with a razor blade. One-half of each liver was placed into a 15 ml conical tube and 1.0 ml of lysis buffer (10 mM Tris, 0.14 M NaCl, pH 8.6) was added. The tissue was homogenized using a 1 ml syringe and a 20 gauge needle. Next, 1.0 ml of 2x PK buffer (200 mM Tris pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% (w/v) SDS, and 500  $\mu$ g/ml proteinase K) was added. The

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tube was inverted several times, then incubated at 37°C overnight. The samples were extracted twice with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). DNA was ethanol precipitated, washed with 70% ethanol, and resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA.

A Southern analysis was performed to quantitate the levels of vector in the liver. Ten micrograms of each DNA sample were cut with BamHI. The digested DNA samples were subjected to electrophoresis in an 0.8% Seakem agarose gel in 40 mM Tris, 20 mM NaAcetate, 1 mM EDTA, pH 7.5.

After electrophoresis, the gel was treated with 0.2 N NaOH, 0.6 M NaCl for 1 hour, then neutralized with 1 M Tris pH 7.4, 0.6 M NaCl for 30 minutes. The DNA was transferred to a nylon membrane by blotting in 10 x SSC. The nylon membrane was baked at 80°C for 1 hour in a vacuum oven. It was prehybridized for 3 hours at 42°C in 5x Denhardt's, 5x SSC, 50 mM NaPhosphate pH 6.5, 250 µg/ml salmon sperm DNA, 0.1% SDS, and 50% formamide. The membrane was hybridized for 24 hours at 42°C in 1x Denhardt's, 5x SSC, 20 mM NaPhosphate pH 6.5, 100 µg/ml salmon sperm DNA, 0.1% SDS, 50% formamide, and 33 µCi of random primer labeled human Factor IX cDNA. Random primer labeling was performed using the BRL kit. The membrane was washed in 2x SSC, 0.1% SDS for 20 minutes at room temperature, followed by a 30 minute wash in 2x SSC, 0.1% SDS at 50°C, and then a 30 minute wash in 0.1x SSC, 0.1% SDS at 68°C. The membrane was exposed to film for 16 hours, then developed. A copy of the autoradiogram is shown in Figure 15. All three routes of administration yielded the same results. The Factor IX bands were the appropriate size with an intensity that indicated an average of 5-10 copies per liver cell.

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Example 3In vivo expression of Factor IX in mice injected with AvlH9B

A second large scale virus preparation of AvlH9B was performed using the same protocol described above, except that 28 15 cm plates of 293 cells were used to amplify the virus. This preparation yielded a much thicker opalescent band upon CsCl gradient centrifugation than the first virus preparation. The titer of this virus preparation was  $1.1 \times 10^{11}$  pfu/ml.

A second in vivo experiment, designed to follow the time course of expression, was initiated using the new AvlH9B preparation. Virus was administered to mice as described above, except that 0.1 ml of a virus suspension ( $1 \times 10^{10}$  pfu) was diluted to 1.0 ml with infection medium. Twenty-seven mice received an injection of recombinant adenovirus: 20 mice received a tail vein injection, 18 with AvlH9B and 2 with AvllacZ4 (encoding  $\beta$ -galactosidase), 4 mice received an intraparenchymal injection of the liver, and 3 received an intramuscular injection. A negative control mouse was not injected. The animals were bled once a week for seven weeks. Plasma levels of human Factor IX are shown in Table I.

As shown in Table I, IP means intraparenchymal injection of AvlH9B, TV means a tail vein injection of AvlH9B, IM means an intramuscular injection of AvlH9B, LacZ means a tail vein injection of AvllacZ4, and NI means no injection (control).

Table Ing/ml Factor IX in plasma

<u>Mouse</u>	<u>Injection</u>	<u>Week 1</u>	<u>Week 2</u>	<u>Week 3</u>	<u>Week 4</u>
1.	TV	376	475	281	171
2.	TV	270	500	392	336
3.	TV	229	374	---	---
4.	TV	240	---	---	---
5.	TV	362	---	---	---
6.	TV	346	---	---	---



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Table I (continued)

		<u>ng/ml Factor IX in plasma</u>			
7.	TV	303	422	252	142
8.	TV	260	573	394	220
9.	TV	353	376	273	149
10.	TV	321	357	270	246
11.	TV	431	482	233	203
12.	TV	347	332	---	---
13.	TV	135	244	126	61
14.	TV	261	294	187	148
15.	TV	212	269	132	91
16.	TV	207	255	214	176
17.	TV	278	218	151	149
18.	TV	170	308	---	---
19.	IM	0.9	3.0	0.0	0.0
20.	IM	1.0	2.7	0.0	0.0
21.	IM	1.1	2.6	1.0	1.2
22.	IP	364	316	174	131
23.	IP	211	308	134	66
24.	IP	305	252	155	206
25.	IP	527	406	133	94
26.	LacZ	0.0	2.8	1.5	0.4
27.	LacZ	0.0	2.6	2.0	1.2
28.	NI	0.0	2.6	1.5	0.5
<u>Mouse</u>	<u>Injection</u>	<u>Week 5</u>	<u>Week 6</u>	<u>Week 7</u>	
1.	TV	98	34	16	
2.	TV	187	67	16	
3.	TV	---	---	---	
4.	TV	---	---	---	
5.	TV	---	---	---	
6.	TV	---	---	---	
7.	TV	---	---	---	
8.	TV	197	60	25	
9.	TV	131	90	46	
10.	TV	179	76	16	
11.	TV	---	---	---	

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12.	TV	---	---	---
13.	TV	84	62	17
14.	TV	133	65	26
15.	TV	94	64	33
16.	TV	---	---	---
17.	TV	92	57	23
18.	TV	---	---	---
19.	IM	0.0	2.0	0.0
20.	IM	0.0	2.0	4.1
21.	IM	0.1	2.0	0.0
22.	IP	112	53	48
23.	IP	42	28	19
24.	IP	299	203	154
25.	IP	57	21	13
26.	LacZ	0.0	1.5	5.0
27.	LacZ	0.0	1.5	4.0
28.	NI	1.4	1.7	0.5

Example 4Assay for biological activity of human Factor IX

The biological activity of human Factor IX in mouse plasma was determined by using an immunocapture, chromogenic assay. A 96-well microtiter plate was coated with a BGIX1 monoclonal antibody obtained from Elcotech, Inc., which recognizes, but does not inactivate, human Factor IX. Coating was done by adding 100 $\mu$ l of a 10 $\mu$ g/ml suspension of the antibody to each well and incubating at room temperature overnight. Plasma samples obtained from Mouse 7 in Example 3 two weeks after injection with the recombinant adenovirus (100 $\mu$ l of 1:5 and 1:10 dilutions) were added to the wells and human Factor IX was allowed to bind. The wells were washed to remove unbound material, and captured human Factor IX was activated by adding 100 $\mu$ l of a 2 $\mu$ g/ml suspension of Factor XIa (Enzyme Research Labs) and incubating at 37°C for 30 minutes. The wells were washed, and then 100 $\mu$ l of a mixture containing 5.0 $\mu$ g phospholipid (Kabi Pharmacia, Franklin, Ohio), 0.1 unit Factor X (Kabi), 0.5 unit Factor VIII (Elcotech), 3.4 $\mu$ g I-2581 thrombin inhibitor (Kabi) and 2.5mM CaCl<sub>2</sub> were added. The plate was incubated at 37°C for 30 minutes, during which time Factor X was converted to Factor Xa. 100 $\mu$ l of 0.5mM N-N-alpha-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-p-nitroanilide-dihydro chloride, a chromogenic Factor Xa substrate, then was added and the plate was incubated at room temperature for ten minutes. The color development was stopped by adding 50 $\mu$ l of 50% acetic acid. The absorbance at 405nm was determined using a Bio-Rad microplate reader. Standard curves (log-log and linear-linear) were generated using normal pooled human plasma, assuming Factor IX levels of 5000ng/ml. Biologically active Factor IX was determined to be 511ng/ml according to the log-log method, and 415ng/ml according to the linear-linear method. Such results are within experimental error, and indicate that essentially all of

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the total Factor IX antigen determined in Example 3 (422ng/ml) is biologically active.

### Example 5

#### Construction of Adenoviral Vector Including DNA Encoding a Factor VIII Derivative

pAVS6H81 was constructed from pMT2LA (Figure 16) and pAVS6. (Figure 10). pMT2LA (Genetics Institute, Cambridge, Mass.) includes cDNA encoding a derivative of human Factor VIII in which the B domain of Factor VIII is deleted. Such cDNA is further described in Toole, et al., Nature, Vol. 312, pgs. 342-349 (November 1984), Vehar, et al., Nature, Vol. 312, pgs. 337-342 (November 1984), and Toole, et al., PNAS, Vol. 83, pgs. 5939-5942 (August 1986). The cDNA is controlled by a Rous Sarcoma Virus promoter. The 4.6 kb cDNA (Figure 17) contains no natural 5' untranslated DNA, and 216 bp of 3' untranslated DNA. The B domain deletion removes nucleotides 2334-4973 of the coding sequence of the full length Factor VIII. The cDNA for B domain deleted Factor VIII could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,868,112.

The cDNA was excised from the plasmid pMT2LA by restriction digestion with XhoI and SalI. The ends were filled in using Klenow, and the fragment encoding the Factor VIII derivative was isolated on an 0.8% agarose gel, followed by electroelution. This fragment was subcloned into the EcoRV site of pAVS6 (Figure 10) to generate pAVS6H81. (Figure 18.)

The recombinant adenoviral vector AvlH81 is generated as depicted in Figure 19.  $1.5 \times 10^6$  293 cells are cotransfected in a 60 mm tissue culture dish with 4  $\mu$ g of the large ClaI fragment of Ad dl 327 and 5  $\mu$ g of pAVS6H81 digested with NotI. Transfections are done using BRL's Transfinity calcium phosphate transfection system. Approximately 15 hours after transfection, medium containing DNA/calcium phosphate precipitate is removed

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from the dishes, the cells are gently washed with PBS, then overlaid with a mixture of 2x MEM and 2% Sea Plaque agarose.

Recombinant adenovirus can be prepared from plaques and analyzed by PCR for the presence of human Factor VIII cDNA.

#### Example 6

##### Generation of Adenoviral Vectors Including DNA Encoding Factor VIII Plus Genomic Elements

##### A. Construction of pAVALH81

The mouse albumin promoter (Zaret, et al., Proc. Nat. Acad. Sci. USA, Vol. 85, pgs. 9076-9080 (1988)), containing 3.5 copies of a liver-specific transcription factor binding site (eG binding sites, Liu et. al., Mol. Cell. Biol., Vol. 11, pgs. 773-784 (1991) and Di Persio, et al., Mol. Cell. Biol., Vol. 11, pgs. 4405-4414 (1991)) was PCR amplified from pAT2-3eG (Figure 20, provided by Kenneth Zaret, Brown University, Providence, Rhode Island) using oligo MGM8.293,

5'-GGC TAG ACG CGT GCT ATG ACC ATG ATT ACG AA-3'

complementary to nts 4281-4299 of pAT2-3eG with the addition of an MluI restriction site, as the 5' oligo, and oligo MGM5.293,

5'-GGT ACG GAT CCA TCG ATG TCG ACG CCG GAA AGG TGA TCT GTGT-3'

complementary to nts 5231-5212 of pAT2-3eG with the addition of BamHI, ClaI, and SalI restriction sites, as the 3' oligo. The PCR product was cut with MluI and BamHI and inserted into pAVS6 (Figure 10) cut with MluI and BamHI to generate pAVAL1 (Figure 21). The sequence of the 964 bp PCR-generated albumin promoter has been verified by sequencing. In addition, at least 50 bp on either side of the MluI site (nt 428) and BamHI site (nt 1392) in

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pAVAL1 (Figure 21) have also been verified by sequencing. The plasmid pAT-2-3eG is prepared according to the procedures disclosed in DiPersio, et al., Mol. Cell. Biol., 11:4405-4414 (1991) and Zaret, et al., Proc. Nat. Acad. Sci., Vol. 85, pgs. 9076-9080 (1988), which disclose the preparation of a mouse albumin promoter with two copies of a liver-specific transcription factor binding site. The plasmid pAT2-3eG has been deposited under the Budapest Treaty in the American Type Culture Collection, 1230 Parklawn Drive, Rockville, Maryland 20892, and assigned accession number 69603.

The ITR, encapsidation signal (see construction of pAVS6) and the albumin promoter were removed from pAVAL1 by digestion with NotI (the ends were filled in with T4 DNA polymerase) and SalI, and inserted into pGEM(sac) (Figure 22), cut with SalI and SmaI to generate pGEMalb (Figure 24) (pGEM(sac) was created by cutting pGEM (Figure 23, Promega) with SacI, and blunting the ends with T4 DNA polymerase and religation, thereby removing the SacI site.) A 1914 bp fragment, containing the 5' region of the B-domain deleted factor VIII cDNA was isolated from pMT2LA (Figure 16) by digestion with BamHI (filling in the 5' end with T4 DNA polymerase) and digestion with XhoI, and inserted into pGEMalb digested with HindIII (filled in with T4 DNA polymerase) and SalI, to generate pGEMalbf8B (Figure 25). pGEMalbf8B was cut with MluI and SpeI, and the resulting 1556 bp fragment was inserted into pAvS6H81 (Figure 18), cut with MluI and SpeI, to generate the adenovirus shuttle plasmid, pAVALH81 (Figure 26). At least 50 bp on either side of the MluI site (nt 429) and SpeI site (nt 1985) have been verified by sequencing of Av1ALH81 viral DNA (see below). The sequence of the Factor VIII B-domain deleted cDNA has been verified by sequencing of bases 1075 to 5732 from the original plasmid, pMT2LA (Figure 16) obtained from Genetics Institute. It should be noted that this sequence differs from the sequence reported by Genetics Institute by two bases. One base change, nt 1317 of pMT2LA was reported by Genetics Institute to be a T (Toole et. al., Nature, Vol. 312,

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pgs. 342-347 (1984) and by Wood et. al., Nature, Vol. 312, pgs. 330-337 (1984) to be an A. In addition, nt 5721 of pMT2LA, reported by Genetics Institute to be a T, was deleted, thus creating a BamHI site within the Factor VIII 3' untranslated region. This mutation does not change the Factor VIII coding region.

#### B. Construction of pAvAPH81

A 1913 bp fragment was isolated from pAVS6H81 (Figure 18) by digestion with Bam HI, and inserted into pGEM(sac) (Figure 22) cut with Bam HI, to create pGemF8B2 (Figure 27). The ApoA1 promoter, first exon (untranslated), first intron, and second exon to the ATG (Genbank #X07496) were PCR amplified using pBGS19-AIgi (Figure 28) as the template. pBGS19-AIgi (Figure 28) was constructed in two steps: 1) The 13 kb SalI fragment was removed from Lambda Al 103 (Swanson, et. al., Transgenic Research, Vol. 1, pgs. 142-147 (1992), and inserted into pUC19 (Figure 29, Gibco BRL) to generate pUC19-AIgi (Figure 30). 2) The 2 kb SmaI fragment was isolated from pUC19-AIgi (Figure 30) and inserted into pBGS19 (Figure 31) to generate pBGS19-AIgi (Figure 28). pBGS19 (ATCC No. 37437) is a kanamycin analog of pUC19. PCR-amplification of pBGS19-AIgi was performed using oligo SSC1.593,

5'GCT CTA GAA CGC GTC GGT ACC CGG GAG ACC TGC AAG CC-3'  
complementary to bases 5862 to 16 of pBGS19-AIgi, containing an XbaI and a MluI site, as the 5' oligo, and a 3' oligo SSC2.593,

5'-GGA ATT CGA GCT CTAT TTG CAT CCT GAA GGG CCG TGG GGA CCT  
GG-3'

complementary to human factor VIII (Genbank #K01740, nts 151-165 (to the SacI site), and nts 463-487 of pBGS19-AIgi, complementary to the ApoA1 gene (Genbank #X07496) with the addition of a SacI and an EcoRI site. The PCR fragment was digested with XbaI and SacI and the resulting 509 bp fragment was inserted into pGemF8B2

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(Figure 27) digested with XbaI-SacI, to generate pGemAPF8B (Figure 32). pGemAPF8B was then digested with MluI-SpeI, and the resulting 1084 bp fragment was ligated into pAVS6H81 (Figure 18) cut with MluI and SpeI, to generate the shuttle plasmid, pAvAPH81 (Figure 33). The sequence of pAvAPH81, from nts 290 to 1619, which include the PCR-generated ApoA1 promoter region, and all cloning junctions, has been verified.

#### C. Construction of pAVALAPH81

A SalI site was added upstream from the ApoA1 transcription initiation site by PCR amplification of pGemAPF8B (Figure 32) using a 5' oligo SSC3.593,

5'-GAA TTC GTC GAC AGA GAC TGC GAG AAG GAG GTG CG-3'  
complementary to the ApoA1 gene (Genebank #X07496) and nts 252-274 of pBGS19-Alg1 (Figure 28) with the addition of an EcoRI and a SalI site, and a 3' oligo, SSC2.593 (see above). The PCR fragment was digested with SalI-SacI, and the resulting 250 bp fragment was inserted into pGemF8B2 (Figure 27) cut with SalI-SacI, to create pGemAPexF8B (Figure 34). The plasmid, pALAPF8B (Figure 35) was generated by a 3-way ligation of the 953 bp MluI-SalI fragment isolated from pGEMalb (Figure 24), the 825 bp SalI-SpeI fragment isolated from pGemAPexF8B (Figure 34), inserted into pGemAPF8B (Figure 32) cut with MluI-SpeI. The 1778 bp MluI-SpeI fragment was isolated from pALAPF8B (Figure 35) and inserted into pAVS6H81 (Figure 18) to generate the shuttle plasmid, pAVALAPH81 (Figure 36).

#### D. Generation of recombinant adenovirus vectors

The recombinant adenoviral vector, Av1ALH81, was generated as outlined in Figure 37.  $2 \times 10^6$  293 cells were cotransfected in a 100 mm tissue culture dish with 10  $\mu$ g of the large ClaI fragment of Ad-dl327, and 10  $\mu$ g of the undigested shuttle plasmid, pAVALH81 (Figure 26). Transfections were performed



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using the Transfinity calcium phosphate transfection system from BRL. Approximately 12 hrs after DNA addition, the cells were washed 2X with 1X PBS, then overlaid with a 1:1 mixture of 2X MEM (GIBCO'S 2X Modified Eagle Medium supplemented with 15% FBS) and 2% SeaPlaque agarose.

Plaques were harvested with sterile Pasteur pipettes and transferred into 0.1 ml of infection medium (Improved Minimum Essential Medium ([IMEM], 2% FBS) in an Eppendorf tube, and subjected to three rounds of freeze/thaw cycles. Cell debris was removed by a 15 second centrifugation at full speed in a microfuge.

Plaques were screened for the presence of recombinant adenovirus as follows. Approximately  $5 \times 10^5$  293 cells were seeded per well of 6-well tissue culture plates. The following day, media was removed from the cells and replaced with 0.4 ml of infection medium and 0.05 ml of the resuspended plaque. The plates were incubated with rocking, for 90 min. in a 37°C/5% CO<sub>2</sub> incubator, after which 2 ml of complete medium (IMEM, 10% FBS) were added. When the cytopathic effect (CPE) was complete, cells were rounded and becoming detached from the plate (approximately 40-120 hrs after infection), cells and medium were transferred to 15 ml conical tubes, and centrifuged at 1000 rpm for 5 min. to pellet cells. The medium was removed from the cell pellet, and the cells were processed as follows.

Cells were resuspended in 250 µl of PK buffer (5mM Tris pH 8.0, 5mM EDTA, pH 8.0, and 0.5% SDS) plus 250 µl of Proteinase K (1mg/ml), and incubated 4 hrs or overnight at 37°C. The solution was transferred to Eppendorf tubes and extracted with an equal volume of phenol 1X, phenol-CHCl<sub>3</sub> 1X, and CHCl<sub>3</sub> 1X, and ethanol precipitated. Pellets were resuspended in 50 µl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0), and genomic DNA was analyzed by restriction digestion. One plaque yielded the expected product.

This plaque of Av1ALH81 was plaque purified as follows.  $5 \times 10^5$  293 cells per well were plated on a 6-well tissue culture

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plate. The next day, medium was removed from the cells, and 0.4 ml of infection medium containing 3 varying amounts of the resuspended plaque were added to each well, 25  $\mu$ l, 2.5  $\mu$ l, and 0.25  $\mu$ l. The plate was rocked for 1.5 hrs in a 37°C/5% CO<sub>2</sub> incubator, after which the media was removed, and the wells were overlaid with a 1:1 mixture of 2X MEM and 2% SeaPlaque agarose as described. Plaques were visible in all wells 9 days after infection. Several plaques were picked from the lowest dilution well (0.25  $\mu$ l of resuspended plaque), and screened for the presence of Av1ALH81 as described. All plaques yielded the expected virus.

One plaque-purified plaque was selected for large scale virus preparation. 5 x 10<sup>5</sup> cells were plated in each well of a 6 well plate and the next day infected with 50  $\mu$ l of the resuspended plaque-purified plaque as described. Five days after infection, the CPE was complete, cells and medium were transferred to 15 ml conical tubes and subjected to four freeze/thaw cycles, then cleared of cell debris by centrifugation at 1000 rpm for 5 min. The resulting supernatant is referred to as crude viral lysate #1 (CVL-1). This CVL was used to infect a 150 mm plate containing approximately 2 x 10<sup>7</sup> 293 cells as follows.

Medium was replaced with 1.25 ml of Infection Medium plus 100  $\mu$ l of CVL, and the plate was rocked for 1.5 hrs as described, after which 20 mls of complete medium was added. Approximately 20 hrs after infection, the CPE was complete, and cells and medium were transferred to a 50 ml conical tube, spun for 5 min at 1000 rpm, supernatant was removed and saved, and the cell pellet was resuspended in 5 ml of supernatant. After four freeze/thaw cycles, the CVL was removed of cell debris as described. The resulting supernatant is referred to as CVL-2. 30-80% confluent 150 mm plates of 293 cells were infected using the CVL-2 as follows.

600  $\mu$ l of CVL-2 was added to 38 mls of Infection Medium, medium was removed from the plates, and replaced with 1.25 ml of

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the CVL-2-Infection Medium mixture. Plates were rocked for 1.5 hrs as described, after which 20 mls of complete medium was added to each plate. The CPE was complete after 30 hrs and cells were processed as follows. Cells and media were harvested into 250 ml centrifuge bottles and spun at 1500 rpm for 10 min. The cell pellet was resuspended in 20 mls of supernatant. Cells were lysed by five freeze/thaw cycles, followed by centrifugation in a SW40 rotor at 7000 rpm for 10 min at 4°C. Virus in the supernatant was purified on a CsCl step gradient as follows.

3.0 ml of 1.25 g/ml CsCl in TD buffer (25 mM Tris, 137 mM NaCl, 5mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, Ph 7.5) was placed in four ultraclear Beckmann #344060 ultracentrifuge tubes. 3.0 ml of 1.4 g/ml CsCl in TD buffer was then underlaid. The CsCl layers were overlaid with 5.0 ml of viral supernatant. Centrifugation was performed at 35,000 rpm, 22°C for 1 hr in a SW40 rotor. Two bands were visible, an upper band consisting of empty capsids, and a lower band composed of intact recombinant adenovirus.

The lower band was collected with a 3 ml syringe and a 18 gauge needle, and then rebanded by placing 8.0 ml of 1.33 g/ml CsCl in TD buffer into two ultracentrifuge tubes, and overlaying with virus collected from the first spin. Centrifugation was performed at 35,000 rpm, 22°C for 18 hrs. The viral band was collected as described and glycerol was added to a final concentration of 10%. The virus was dialyzed against one liter of 10 Mm Tris pH 7.4, 10 Mm MgCl<sub>2</sub>, and 10% glycerol at 4°C. Dialysis lasted for four hours with buffer changes every hour. The virus was recovered and stored at -70°C in aliquots in sterile Eppendorf tubes. The titer of this virus preparation (Lot # MS1-1) was  $1.5 \times 10^{11}$  pfu/ml. A second Av1ALH81 viral prep was made in a similar manner as described, again using 600 µl of CVL-2 and 30-150 mm plates of 80% confluent 293 cells. The titer of the second prep (Lot # MS1-2) was  $9 \times 10^{10}$  pfu/ml.

At this stage, the viral DNA is checked for deletions or rearrangements. Studies utilizing retroviral vectors containing Factor VIII cDNA sequences have been shown to delete and/or

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rearrange portions of the Factor VIII CDNA at high frequencies (Lynch et. al., 1993), and similar rearrangements may be seen with Factor VIII-containing adenoviral vectors. Therefore, viral DNA was isolated from both lots (MS1-1, and MS1-2) of Av1ALH81 as follows. 100  $\mu$ l of purified virus was added to 100  $\mu$ l of TE, 5  $\mu$ l of 10% SDS, and 20  $\mu$ l of 10 mg/ml Proteinase K (Sigma), and digested overnight at 37°C. The viral DNA was extracted with an equal volume of phenol 1X, phenol-CHCl<sub>3</sub> 1X, and CHCl<sub>3</sub> 1X, then the supernatant was put over a Centricon 10 concentrator (Amicon) and the volume was increased to 2 mls with TE, and spun at 5000 rpm for one hour. The centricon was then washed with 2 mls of TE, and spun for 30 min at 5000 rpm. DNA was recovered by inverting the upper chamber of the centricon, inserting into the collection tube, and centrifugation at 3000 rpm for 5 min. Final volume of the purified DNA was increased to 100  $\mu$ l, and the DNA concentration was calculated. 10  $\mu$ g of MS1-1, MS1-2, and dl327 DNA was digested overnight with BamHI, HindIII, or, NdeI, and run on a 0.8% agarose gel. DNA fragments were visualized with ethidium bromide staining (Figure 38). Both Av1ALH81 lots look the same, and all restriction fragments are of the predicted sizes. Therefore, unlike the Factor VIII-containing retroviral vectors (Lynch et. al., 1993), the genome of Av1ALH81 is stable.

The recombinant adenoviral vector Av1ALAPH81 was generated as outlined in Figure 39.  $2 \times 10^6$  293 cells were cotransfected in a 100 mm tissue culture dish with 10  $\mu$ g of the undigested shuttle plasmid, pAv1ALAPH81 (Figure 36). Generation of adenoviral vector Av1ALAPH81 then was carried out in the same manner as the generation of adenoviral vector Av1ALH81. Av1APH81 can be generated in the same manner.

#### Example 7

#### In Vivo Expression of Adenoviral Vectors Including DNA Encoding Factor VIII Plus Genomic Elements

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A. Factor VIII Tri-Sandwich ELISA

Before Av1ALH81 could be tested for Factor VIII expression *in vivo*, in mice, or *in vitro*, in tissue culture cells, it was necessary to develop an assay capable of measuring low levels of human Factor VIII present in mouse plasma. The only commercially available Factor VIII assay, Coatest (Kabi Pharmaceuticals) measures the biological activity of Factor VIII protein, and can be used to measure Factor VIII levels in tissue culture cells. However, Coatest cannot distinguish human Factor VIII from animal Factor VIII and, therefore, is not useful for measuring human Factor VIII in animal plasma. To measure the amount of human Factor VIII present in tissue culture medium or animal plasma samples, a quantitative Factor VIII tri-sandwich ELISA was developed. This ELISA can measure human Factor VIII specifically in mouse and dog plasma, and can measure reproducibly Factor VIII concentrations down to 1.0 ng/ml. The assay is performed as follows.

A 96 well microtiter plate is coated with two commercially available monoclonal antibodies with unique epitopes for Factor VIII protein and incubated overnight at 4°C to allow adherence to the plastic wells. 0.5 ug of each antibody (N7, Biodesign; and ESH2, American Diagnostica) were diluted in dilution buffer (1.59 g Na<sub>2</sub>CO<sub>3</sub>, 2.93 g NaHCO<sub>3</sub>, sterile H<sub>2</sub>O to one liter, pH 9.6), and 100 µl of the dilution was added to each well. These antibodies constitute the primary antibody. The use of two capture antibodies, that act cooperatively to increase the sensitivity of the assay, has not been described previously. After the overnight incubation, the plate is washed gently 3X with 200 µl per well of 1X PBS and blotted dry. Blocking agent [1X PBS, 10% Horse Serum (heat inactivated, BioWhittaker), and 1 mM CaCl<sub>2</sub>] is added, and incubated for two hours at room temperature, after which the plate is washed with 200 µl per well of washing solution [1X PBS, 0.05% Tween 20 (Sigma)] 3X and blotted dry. Samples then are diluted appropriately (usually a 5-fold

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dilution) in TNTC (50 mM Tris pH 7.2, 5 mM CaCl<sub>2</sub>, 0.1% Tween 20, 0.5 M NaCl), aliquoted into each well, and incubated for one hour at 37°C, after which the wells are washed with the washing solution as described. The secondary antibody, which is diluted serum from a hemophiliac (a 1:1000 dilution in the blocking agent solution, 100 µl per well) containing Factor VIII inhibitor antibodies, is added and allowed to bind for one hour at 37°C, after which the wells are washed with the washing solution as described. The third antibody, a commercially available goat anti-human IgG antibody conjugated to horseradish peroxidase (goat anti-human IgG-HRP, Pierce, 0.8 mg/ml, diluted 1:5000 in blocking agent, 100 µl per well), is added, and incubated for one hour at 37°C. The excess antibody then is washed out of the wells (as described, but 5X) and the substrate tetramethylbenzidine (TMB) (Kirkegaard and Perry Labs; 100 µl of the commercially available solution), which when cleaved by the HRP, yields a blue color, is added to each well. The level of color that develops is proportional to the amount of Factor VIII present in the sample. The reaction is stopped, after 2-3 minutes with an acid stop solution (TMB stop solution, Kirkegaard and Perry Labs, 100 µl per well) and the absorbance is determined using a microtiter plate reader. An example of a typical standard curve, using full-length human Factor VIII protein concentrations ranging from 0.078 ng/ml to 40.00 ng/ml is displayed in Figure 40.

B. Half-Life Study of B-Domain Deleted Factor VIII in Mouse Plasma

After development of this extremely sensitive Factor VIII ELISA, a half-life study of B-domain deleted (BDD) Factor VIII in mouse plasma was undertaken. It had been reported (Hoeben et al., 1993) that the half-life of human Factor VIII in mice was only one hour, compared to the 10 to 12 hour half-life of full-length human Factor VIII in humans and dogs (Brinkhous, et al.,

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PNAS, Vol. 82, pgs. 8752-8756 (1985)). The determination of the half-life of BDD human Factor VIII in mice was important for the subsequent evaluation of the efficacy of Av1ALH81 for gene therapy protocols utilizing the mouse as an *in vivo* model.

The half-life study was performed twice. In the first experiment (Figure 41) five C57bl/6 female mice were injected via tail vein with 400 ng of BDD Factor VIII protein. Blood was drawn at 0.5, 1.5, 2.5, and 6.5 hours post injection. In the second experiment (Figure 42), designed to focus on the 2 to 14 hour post injection time range, four C57bl/6 female mice were injected via tail vein, with 500 ng of BDD Factor VIII. Blood was drawn at 2, 5, 8, 12, and 14 hours post injection and plasma analyzed for the presence of human Factor VIII antigen. The results are displayed in Figures 41 and 42. The half-life of human Factor VIII in mice was calculated to be 4-5 hours. This result contrasts with the half-life calculated by Hoebe et. al. (1993). However, in the study by Hoebe et. al. (1993), the half-life of Factor VIII in mice was analyzed over only a 2 hour time period. In the study reported here, it was found that there was a sharp decrease (half-life 1.7 hours) in the level of Factor VIII antigen in mouse plasma between 30 minutes and 2 hours post injection (Figure 41), with the decay leveling off to a half-life of 4-5 hours at subsequent time points (Figures 41 and 42). Therefore, the results indicate that the half-life of human BDD Factor VIII in mice is approximately 2-3 times shorter than the human Factor VIII half-life in humans and dogs.

#### C. Production of Biologically Active Factor VIII In Vitro

To determine if Av1ALH81 transduction resulted in the production of biologically active Factor VIII *in vitro*, 293 cells were infected with CVL-1, generated from two separate plaques of plaque-purified Av1ALH81 as follows. The medium was removed from 3-150 mm plates of 293 cells containing  $1.5 \times 10^7$  cells, and replaced with 1.15 mls of Infection Medium, plus 100  $\mu$ l of CVL-1

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from either Av1ALH81 plaque (plaque 1 or plaque 2), or, for the negative control plate, 1.25 mls of Infection Medium. Plates were rocked for 1.5 hrs. as described, after which 20 mls of complete medium was added to each plate. 1.0 ml of medium was collected from each plate at 0, 12, and 24 hr. time points, and analyzed for the presence of Factor VIII antigen, using the human Factor VIII-specific ELISA, described above, and analyzed for biological activity, using the Coatest Assay (Kabi Pharmaceuticals). The results are displayed in Table II below.

Table II

Expression of Factor VIII in Av1ALH81  
Transduced 293 Cells

<u>Virus</u>	<u>Time (hrs)</u>	<u>Assay</u>	
		<u>ELISA (ng/ml)</u> <u>total antigen</u>	<u>Coatest (ng/ml)*</u> <u>biological activity</u>
Av1ALH81 plaque 1	0	0.0	0.0
	12	9.8	6.9
	24	10.2	1.0
Av1ALH81 plaque 2	0	0.0	0.0
	12	22.1	7.6
	24	24.3	0.0
No virus	0	0.0	0.0
	12	0.0	0.0
	24	0.0	0.0

\* converted from units in which one unit of activity equals 200ng/ml of Factor VIII.

As shown in Table II, the cells produced 10-20 ng/ml of Factor VIII total antigen as determined by ELISA, and at 12 hrs., 7 ng/ml of Factor VIII was biologically active. However, by 24 hours, the biological activity was lost. The lower level of biologically active Factor VIII at 12 hours and the lack of active Factor VIII at 24 hours can be explained by the fact that the cells were undergoing a cytopathic effect that started at 12



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hours and was complete by 24 hours. Therefore, *de novo* synthesis of Factor VIII had probably begun to decrease at 12 hours and the Factor VIII present in the medium was becoming degraded by 24 hours.

D. In Vivo Expression of BDD Factor VIII From Av1ALH81

To determine if human BDD Factor VIII was expressed from Av1ALH81 *in vivo*, and if so, to follow the time course of Factor VIII expression, 15 C57bl/6 female mice were injected with Av1ALH81. The virus was diluted in injection medium (IMEM + 1% FBS) to a total volume of 0.5 ml. Five mice received a dose of  $1 \times 10^{10}$  pfu (67  $\mu$ l of virus; concentration of  $1.5 \times 10^{11}$  pfu/ml), five mice received a dose of  $4 \times 10^9$  pfu (27  $\mu$ l virus) and five mice received  $1 \times 10^9$  pfu (7  $\mu$ l virus). The viral suspension was infused via tail vein over a ten second period using a 0.5 ml syringe and a 27 gauge needle. The control mouse received no injection. One mouse that received  $1 \times 10^{10}$  pfu of Av1ALH81 died two days after injection. Blood was taken from each mouse at one week intervals and analyzed for the presence of human Factor VIII antigen by ELISA. The results of the analysis, for the first five weeks post injection, is displayed in Table III below, and graphically in Figure 43.

Table III  
In Vivo Expression of Factor VIII

<u>Mouse</u>	<u>Virus Dose</u>	<u>ELISA (Factor VIII ng/ml)</u>				
		<u>Week 1</u>	<u>Week 2</u>	<u>Week 3</u>	<u>Week 4</u>	<u>Week 5</u>
1	$1 \times 10^{10}$	53.3	19.3	0.0	4.3	0.0
2	$1 \times 10^{10}$	45.9	54.6	0.0	34.3	0.0
3	$1 \times 10^{10}$	34.5	35.3	3.0	2.6	0.0
4	$1 \times 10^{10}$	33.1	31.1	10.9	7.7	1.8
Mean	-	41.7	35.1	3.5	12.2	0.5
5	$4 \times 10^9$	18.9	7.3	0.0	14.9	1.1
6	$4 \times 10^9$	13.0	6.2	5.1	7.9	3.1
7	$4 \times 10^9$	9.8	5.2	3.1	12.4	5.1
8	$4 \times 10^9$	25.9	13.1	3.4	18.4	12.9
9	$4 \times 10^9$	17.1	5.9	0.5	9.7	6.0
Mean	-	16.9	7.5	2.3	12.6	5.6
10	$1 \times 10^9$	0.8	0.0	0.0	3.6	0.8
11	$1 \times 10^9$	0.4	0.0	0.5	5.1	0.3
12	$1 \times 10^9$	1.1	1.6	2.2	4.2	2.5
13	$1 \times 10^9$	1.0	1.7	0.0	1.7	1.3
14	$1 \times 10^9$	1.4	0.9	0.0	1.9	0.0
Mean	-	0.9	0.9	0.5	3.3	1.0
Control	0	0.0	0.0	0.0	0.0	0.0

The mice receiving the highest viral dose ( $1 \times 10^{10}$  pfu) were producing 42 and 35 ng/ml human BDD Factor VIII one and two weeks post injection, respectively. If these values are corrected for the difference in half-life of human Factor VIII in mice (4-5 hrs., see above) compared to humans (10-12 hrs.), levels in the plasma are adjusted to 126 and 96 ng/ml of Factor VIII at one and two weeks, respectively. Physiological levels of Factor VIII in humans is ~100-200 ng/ml and therapeutic levels are ~10 ng/ml. Therefore, these mice are producing physiological levels of

-50-

Factor VIII. In addition, the mice that received the lower dose of  $4 \times 10^9$  pfu of Av1ALH81 are producing Factor VIII protein well over therapeutic levels. The expression of human Factor VIII, in an animal model, has never before been demonstrated.

Three to five weeks post injection, however, the Factor VIII levels had decreased significantly. To determine if this decrease in Factor VIII expression at three weeks was due to an immunological response to the Factor VIII antigen, a second half-life study was performed using full-length human Factor VIII protein. The four mice that had received the highest viral dose ( $1 \times 10^{10}$  pfu), and four control mice were injected, via tail vein, with 500 ng of full-length human Factor VIII. Blood was drawn at 1, 2, 4, 8, and 12 hours post injection and analyzed for the presence of human Factor VIII antigen by ELISA. Figures 44 and 45 display the results of this analysis. The half-life of full-length Factor VIII is similar in both sets of mice, and can be calculated to be about 3.0 hrs. Two conclusions can be drawn from these data: 1) The half-life of full-length Factor VIII and BDD Factor VIII in mouse plasma are comparable, 3 hrs. and 4-5 hrs., respectively, and 2) the loss in Factor VIII expression in the mice at 3 weeks is not due to rapid clearance of the Factor VIII by specific antibodies in mouse plasma, and, therefore, may be due to the loss of vector from the liver (or loss of the cells containing the vector), or reduced transcription of the Factor VIII cDNA.

The time course of Factor VIII expression in mice was repeated, and the data are shown in Figure 46.

#### Example 8

Fifteen C57bl/6 female mice were injected via tail vein  $4 \times 10^9$  pfu (27  $\mu$ l of virus) of Av1ALH81 in injection medium (IMEM+ 1% FBS). The viral suspension was infused via the tail vein over a ten second period using a 0.5 ml syringe and a 27 gauge needle. A control mouse received no injection. Blood was taken from each

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mouse at one week intervals and analyzed for the presence of human Factor VIII antigen by ELISA. The results are shown graphically in Figure 46.

### Example 9

#### Construction of Adenoviral Vectors Including Factor IX Sequences with Genomic Elements

Vectors were prepared in which the Factor IX sequences incorporated genomic elements, i.e., sequences from the human Factor IX gene. These elements included the 5' untranslated region, a centrally truncated first intron, the full 3' untranslated region and naturally-occurring polyadenylation site. The 5' genomic elements were obtained by PCR amplification using genomic Factor IX clones as templates. The three prime untranslated region was obtained from a plasmid provided by Dr. Hsueh (Shanghai, China). An alternative approach, which can be used to readily obtain these elements, is to PCR amplify them from human genomic DNA.

A Factor IX sequence, which includes 9 bp of the Factor IX promoter, the 5' untranslated region, the coding region, and a 162 bp segment of the 3' untranslated region, was excised from pKG5IX-2 (obtained from George Brownlee, University of Oxford, Oxford England) as a Bam HI to HindIII fragment. This fragment is described further in Anson, et al., Nature, Vol. 315, pgs. 683-685 (1985). This insert was inserted into the polylinker of pBluescript II SK+ (Stratagene) to form BLSKH9CI. (Figure 47). The Factor IX sequences were sequenced completely and verified to be correct. Factor IX DNA with genomic elements could also have been obtained according to the procedures disclosed in U.S. Patent No. 4,994,371 and European Patent EP 0 107 278 B1.

A fragment containing the downstream part of the coding sequence, the full 3' untranslated region, the native Factor IX polyadenylation signal, and 331 bp past the polyadenylation site

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was excised from pCMVIXa (provided by Jerry Hsueh, Fudan University, Shanghai, China) with PpuMI and BglII. The BglII single strand overhang was blunted. pBLSKH9CI was cut with PpuMI and HindIII, the HindIII site was blunted, and the backbone fragment was joined to the fragment obtained from pCMVIXa as a PpuMI-blunt ligation. The resulting plasmid, pBLSKH9D (Figure 48), contains the 9 bp of promoter, 5' untranslated region, the entire Factor IX coding sequence, the full 3' untranslated region, natural polyadenylation signal, and 331 bp downstream from the polyadenylation signal.

To generate constructs that contain a centrally truncated first intron, a cloning intermediate was prepared. This intermediate removed a BclI site downstream from the coding sequence to enable cloning into an upstream BclI site. The 3' end of the cDNA in pBLSKH9CI was removed from PpuMI to HindIII. The single strand overhangs in the plasmid backbone were blunted and ligated to yield pBLH9CINT. (Figure 49)

The 5' end of the cDNA in pBLH9CINT was modified to contain the centrally truncated first intron with a 3 way ligation using 2 PCR generated fragments. These fragments were generated using phage preps as templates (Yoshitake, S, et al., 1985, Biochemistry: 24, 3736-3750). The two PCR generated fragments contained (5' to 3'):

- 1) SpeI site, SalI site, full 5' untranslated region, first exon of the Factor IX gene, the first 991 bp of the Factor IX first intron, and an AatII site.
- 2) AatII site, the last 448 bp of the Factor IX first intron, and part of the Factor IX second exon extending past the naturally occurring BclI site in the upstream part of this exon.

PCR fragment 1 was digested with SpeI and AatII. PCR fragment 2 was digested with AatII and BclI. BLH9CINT was digested with SpeI and BclI and the backbone fragment was isolated. The three fragments were joined with a 3 way ligation to yield the plasmid pBLH9EINT. (Figure 50) This plasmid

-53-

contains the 5' untranslated region of Factor IX, the first exon, the centrally truncated first intron, and the coding sequence up to the PpuMI site.

To generate pBLH9E (Figure 51), the 3' end of the coding sequence was re-inserted. The 3' end of the Factor IX sequence was excised from pBLSKH9CI and inserted into the pBLH9EINT backbone as an *AvaI*-*AvaI* fragment. The resulting plasmid pBLH9E (Figure 51) contained the Factor IX 5' untranslated region, first exon, centrally truncated first intron, remainder of the coding sequence, and 162 bp of 3' untranslated region.

To generate pBLH9F (Figure 52), a fragment containing the 3' end of the coding sequence and the full 3' untranslated region was excised from pBLSKH9D and inserted into the pBLH9EINT backbone as an *AvaI*-*AvaI* fragment. Thus pBLH9F has the 5' untranslated region, first exon, truncated first intron, remainder of the coding sequence, full 3' untranslated region, and 300 bp downstream from the polyadenylation site.

The Factor IX sequences were then excised from pBLSKH9D, pBLH9E, and pBLH9F and inserted into the pAVS6 backbone as *SpeI*-*ClaI* fragments. The resulting plasmids were termed pAV1H9D (Figure 53), pAV1H9E, and pAV1H9F, respectively. However, when pAV1H9E and pAV1H9F were sequenced, errors were found in the 5' untranslated region of the Factor IX gene. These errors were repaired. The sequence errors were traced back to pBLH9EINT. Miniprep one this plasmid had been used to generate the subsequent plasmids. pBLH9EINT miniprep six was found to have the correct sequence. The *SpeI* to *AatII* fragment in pBLH9EINT miniprep six was used to replace the corresponding fragment in pAV1H9E and pAV1H9F to yield pAV1H9ER (Figure 54) and pAV1H9FR (Figure 55), respectively. These plasmids contain the adenovirus type 5 ITR, RSV promoter, tripartite leader, Factor IX sequence, SV40 polyadenylation site (which is superfluous in pAV1H9D and pAV1H9FR), and adenovirus homologous recombination region.

pAV1H9D, pAV1H9ER, and pAV1H9FR were then used to generate adenoviral vectors by procedures hereinabove described. Briefly,

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linearized plasmids were co-transfected with the large ClaI-cut fragment of Ad dl327 into 293 cells. Plaques were selected, expanded, and screened. The three vector isolates chosen were termed Ad1H9D, Ad1H9ER, Ad1H9FR. These were grown into large scale preps and plaque titered on 293 cells.

#### Example 10

$2 \times 10^8$  pfu of Av1H9B, Ad1H9D, Ad1H9ER, or Ad1H9FR were injected via tail vein into C57B1/6 mice. Two mice received each virus. One week later, plasma was sampled by ELISA and an immunochromogenic bioassay described above for human Factor IX antigen and biological activity, respectively. The ELISA results, shown in Figure 56, demonstrate that the inclusion of genomic elements dramatically increased Factor IX expression. The Ad1H9FR vector effected the most expression which was more than 200 fold greater than the level obtained with Av1H9B. The immunochromogenic assay results for eight experimental mice and one negative control mouse (which was injected with the beta-galactosidase vector, Av1LacZ4) are shown in Table IV below.



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Table IV

<u>Vector</u>	<u>Mouse Number</u>	<u>Plasma Factor IX (ng/ml)</u>	
		<u>ELISA</u>	<u>Immunochromogenic</u>
AvlH9B	1	<8	<8
AvlH9B	2	<8	<8
AdlH9D	3	74	65
AdlH9D	4	48	49
AdlH9ER	5	116	102
AdlH9ER	6	101	80
AdlH9FR	7	1,339	1,051
AdlH9FR	8	1,467	1,391
AvlLacZ4	9	<8	<8

These results demonstrate that the human Factor IX expressed from AdlH9D, AdlH9ER, and AdlH9FR was functional. Livers were collected one week after vector injection and DNA and RNA were prepared. Southern analysis demonstrated an average of 1-2 vector copies per liver cell for all four vectors. (Data not shown.) Northern analysis revealed RNA species of the correct size for each vector with band intensities that paralleled the Factor IX plasma levels. (Data not shown.)

Example 11

$1 \times 10^9$  pfu of AvlH9B, AdlH9D, or AdlH9ER were injected via tail vein into C57B1/6 mice.  $5 \times 10^7$  pfu of AdlH9ER and AdlH9FR were also administered to C57B1/6 mice via tail vein injection. The cohort size for each regimen was 5 mice. At the indicated time points plasma was obtained and assayed for human Factor IX by ELISA. The results, shown in Figures 57 and 58, again demonstrate that the inclusion of genomic elements in the Factor IX sequences dramatically increased Factor IX expression. Factor IX levels that approach normal were obtained with the low dose of  $1 \times 10^9$  pfu of AdlH9D and AdlH9ER. Injection of a very low dose

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of  $5 \times 10^7$  pfu of Ad1H9FR resulted in the expression of potentially therapeutic levels of human Factor IX in the mice. In each case, high-level expression from Ad1H9D, Ad1H9ER, and Ad1H9FR persisted for the 10-12 week duration of the experiment. This exceeded the 7 week duration of expression which had previously been achieved with higher doses of Av1H9B.

#### Example 12

Expression from Av1H9B, Ad1H9D, and Ad1H9ER was tested in tissue culture. HepG2 and HeLa cells were transduced at an moi of 2 (2 pfu per cell). 48 hours later the medium was collected and assayed by ELISA for human Factor IX. The data, shown in Figure 59, demonstrate that the incremental improvements seen in HepG2 cells with Ad1H9D and Ad1H9ER correspond to those seen *in vivo* in mice that received  $2 \times 10^8$  pfu of virus. In HeLa cells, the inclusion of the 3' untranslated region (Ad1H9D) had no effect, whereas inclusion of the intron (Ad1H9ER) improved expression dramatically.

All patents, publications, and database entries referenced in this specification are indicative of the level of skill of persons in the art to which the invention pertains. The disclosures of all such patents, publications (including published patent applications), and database entries are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication, and database entry was specifically and individually indicated to be incorporated by reference.

It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

WHAT IS CLAIMED IS:

1. An adenoviral vector including at least one DNA sequence encoding a clotting factor.
2. The vector of Claim 1 wherein said DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof having Factor VIII clotting activity.
3. The vector of Claim 1 wherein said DNA sequence encodes Factor IX or a fragment, derivative, or analogue thereof having Factor IX clotting activity.
4. A plasmid vector for generating an adenoviral vector, said plasmid vector including at least one DNA sequence encoding a clotting factor.
5. A method of treating hemophilia in a host, comprising:  
administering to said host the adenoviral vector of Claim 1, said vector being administered in an amount effective to treat hemophilia in said host.
6. The vector of Claim 2 wherein said vector further includes a tissue-specific promoter.
7. The vector of Claim 6 wherein said tissue-specific promoter is the mouse albumin promoter.
8. The vector of Claim 3 wherein said vector further includes a promoter which is not a tissue-specific promoter.
9. The vector of Claim 8 wherein said promoter which is not a tissue-specific promoter is a Rous Sarcoma Virus promoter.
10. The vector of Claim 3 wherein said vector further includes at least one genomic element.
11. The vector of Claim 10 wherein said genomic element is the full 3' untranslated region of the Factor IX DNA sequence.
12. The vector of Claim 11 wherein said vector further includes the full 5' untranslated region of the Factor IX DNA sequence.
13. The vector of Claim 10 wherein said vector includes the full 3' untranslated region of the Factor IX DNA sequence, the full 5' untranslated region of the Factor IX DNA sequence, and a centrally truncated first intron of the Factor IX gene.

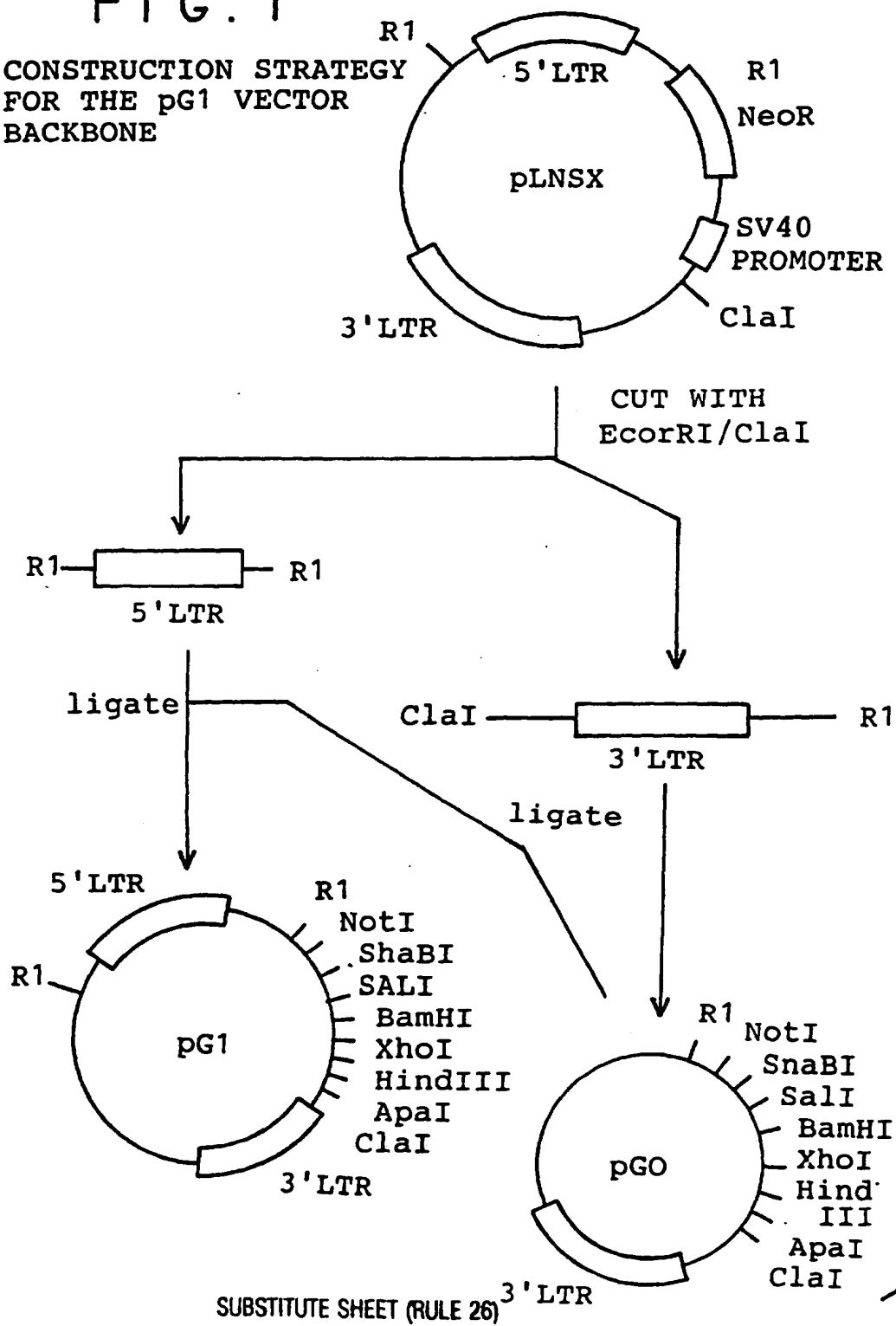
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14. The vector of Claim 10 wherein said vector further includes the full seventh intron of the Factor IX gene.
15. The vector of Claim 2 wherein said vector further includes at least one genomic element.
16. The vector of Claim 15 wherein said vector includes the ApoA1 promoter.
17. The vector of Claim 16 wherein said vector further includes the first intron of the apolipoprotein A-1 gene.
18. The vector of Claim 16 wherein said vector further includes the first exon of the apolipoprotein A-1 gene.
19. The vector of Claim 16 wherein said vector further includes the first intron of the apolipoprotein A-1 gene and the first exon of the apolipoprotein A-1 gene.
20. An adenoviral vector including at least one DNA sequence encoding a heterologous protein, and at least one genomic element affecting expression of said at least one DNA sequence encoding a heterologous protein.

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## FIG. 1

CONSTRUCTION STRATEGY  
FOR THE pG1 VECTOR  
BACKBONE



## FIG. 2

SEQUENCE OF THE MULTIPLE CLONING SITE IN THE pGI PLASMID  
1/2 EcoRI NotI SnaBI SaiI BamHI XhoI HindIII ApaI

AATTC GCGGCCGC TACGTA GTCGAC GGATCC CTCGAG AAGCTT GGGCCC

G CGCCGGCG ATGCAT CAGCTG CCTAGG GAGCTC TTCGAA CCCGGG

1/2 ClaI

AT

TAGC

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## FIG. 11A

1 AGGTTATGCA GCGCGTGAAC ATGATCATGG CAGAATCACC AGGCCTCATC ACCATCTGCC  
 61 TTTTAGGATA TCTACTCAGT GCTGAATGTA CAGTTTCTCT TGATCATGAA AACGCCAACA  
 121 AAATTCTGAA TCGGCCAAAG AGGTATAATT CAGGTAAATT GGAAGAGTTT GTTCAAGGGA  
 181 ACCTTGAGAG AGAATGTATG GAAGAAAAGT GTAGTTTGA AGAAGCACGA GAAGTTTGTG  
 241 AAAACACTGA AAGAACAAC TTTAAATGGC GGCAGTTGCA AGCAGTATGT TGATGGAGAT CAGTGTGAGT  
 301 CCAATCCATG ATTTGAAGGA AAGAACTGTG AATTAGATGT TAATTCCCTAT GAATGTTGGT  
 361 GTCCCTTTGG GCAGATGCCA GCAGTTTGT AAAAATAGTG CTGATAACAA GGTGGTTTGC ATTAAGAATG  
 421 AGGGATATCG ACTTGCAGAA AACCAAGAGT CCTGTGAACC AGCAGTGCCA TCTGTACTG  
 481 GAAGAGTTTC TGTTCACAA ACTTCTAAGC TCACCCGTCG TGAGACTGTT TTTCCATGTG  
 541 TGGACTATGT AAATTCTACT GAAGCTGAAA CCATTTTGGA TAACATCACT CAAAGCACCC  
 601

MATCH WITH FIG. 11B

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MATCH WITH FIG. 11A  
FIG. 11B

661	AATCATTTAA	TGACTTCACT	CGGGTTGTTG	GTGGAGAAGA	TGCCAAACCA	GGTCAATTCC
721	CTTGGCAGGT	TGTTTGAAT	GGTAAAGTTG	ATGCATTCTG	TGGAGGCTCT	ATCGTTAATG
781	AAAAATGGAT	TGTAACCTGCT	GCCCACTGTG	TTGAAACTGG	TGTTAAAAAT	ACAGTTGTCTG
841	CAGGTGAACA	TAATATTGAG	GAGACAGAAC	ATACAGAGCA	AAAGCGAAAT	GTGATTTCGAA
901	TTATTCCCTCA	CCACAACCTAC	AATGCAGCTA	TTAATAAGTA	CAACCATGAC	ATTGCCCTTC
961	TGGAACCTGGA	CGAACCCCTTA	GTGCTAAACA	GCTACGTTAC	ACCTATTTGC	ATTGCTGACA
1021	AGGAATACAC	GAACATCTTC	CTCAAATTG	GATCTGGCTA	TGTAAGTGGC	TGGGGAAGAG
1081	TCTTCCACAA	AGGAGATCA	GCTTTAGTTC	TTCAGTACCT	TAGAGTTCCA	CTTGTGTGACC
1141	GAGCCACATG	TCCTCGATCT	ACAAAGTTCA	CCATCTATAA	CAACATGTTT	TGTGCTGGCT
1201	TCCATGAAGG	AGGTAGAGAT	TCATGTCAAG	GAGATAGTGG	GGGACCCCAT	GTTACTGAAG
1261	TGGAAGGGAC	CAGTTCTTA	ACTGGAATTA	TTAGCTGGGG	TGAAGAGTGT	GCAATGAAAG
1321	GCAAATATGG	AATATATACC	AAGGTATCCC	GGTATGTCAA	CTGGATTAAAG	GAAAAAACAA
1381	AGCTCACTTA	ATGAAAGATG	GATTTCCAAG	GTAAATTTCAT	TGGAATTGAA	AATTAACAGG
1441	GCCTCTCACT	AACTAATCAC	TTTCCCCTCT	TTTGTTAGAT	TTGAATATAT	ACATTCTATG
1501	ATCATTGCTT	TTTCTCTTTA	CAGGGGAGAA	TTTCATATTT	TACCTGAG	

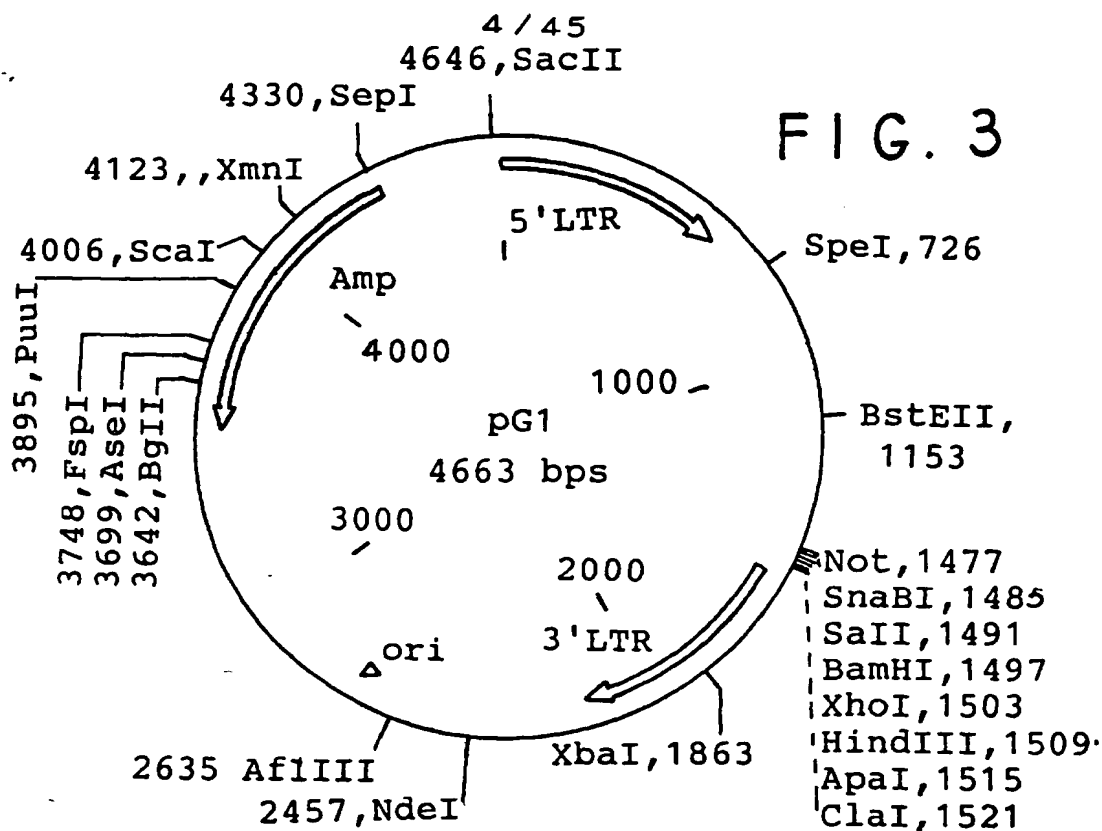


FIG. 3

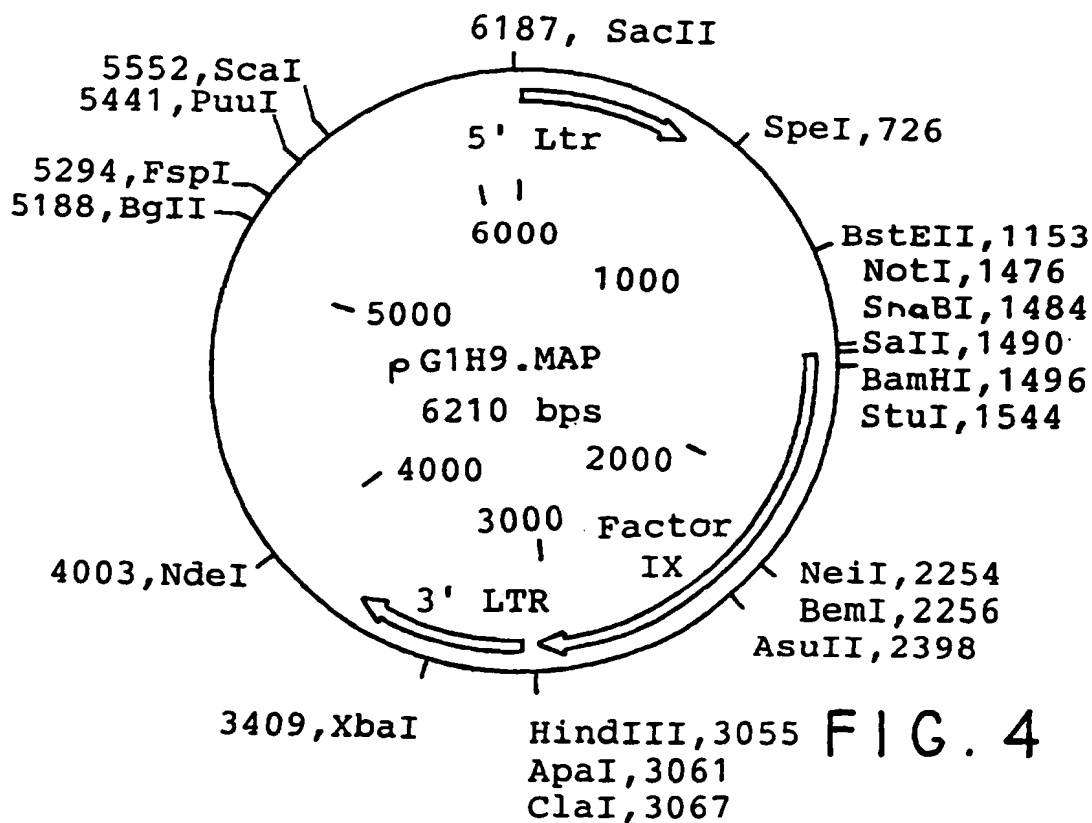
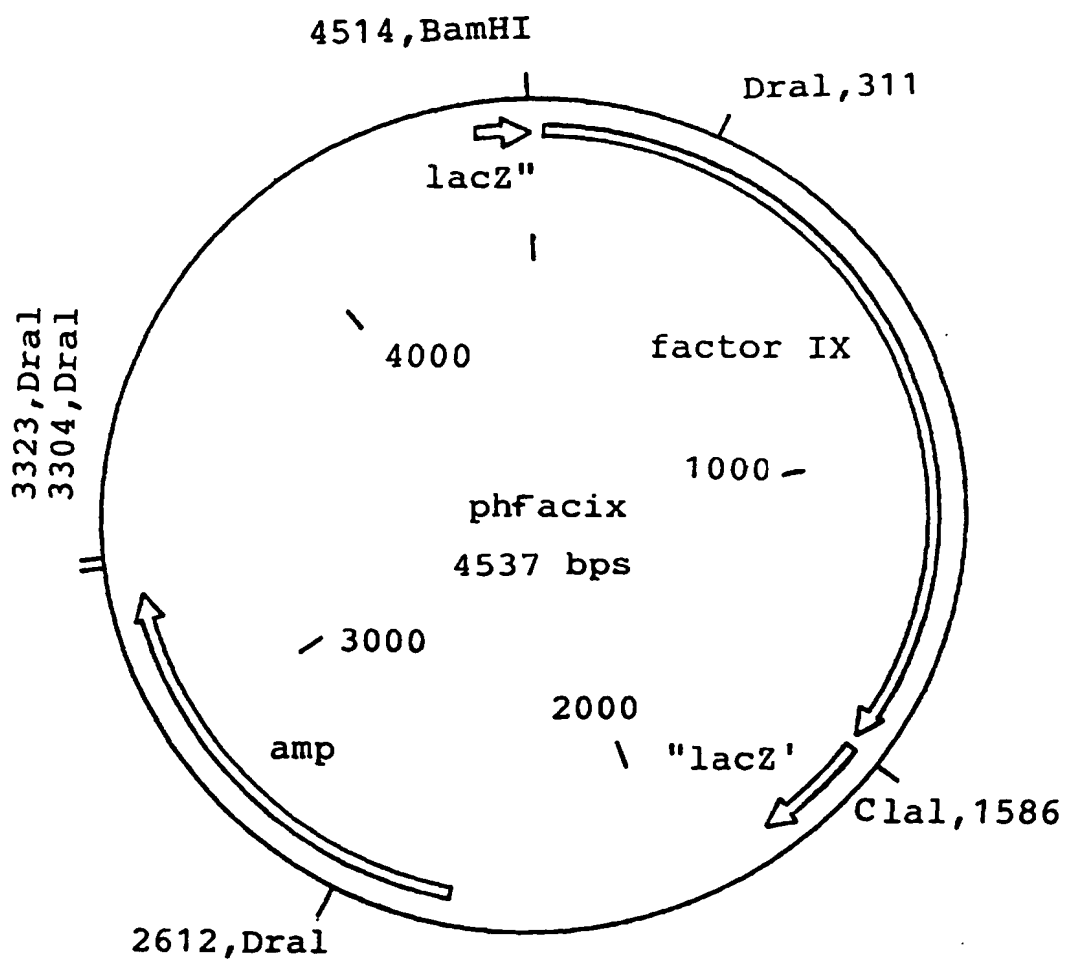


FIG. 4



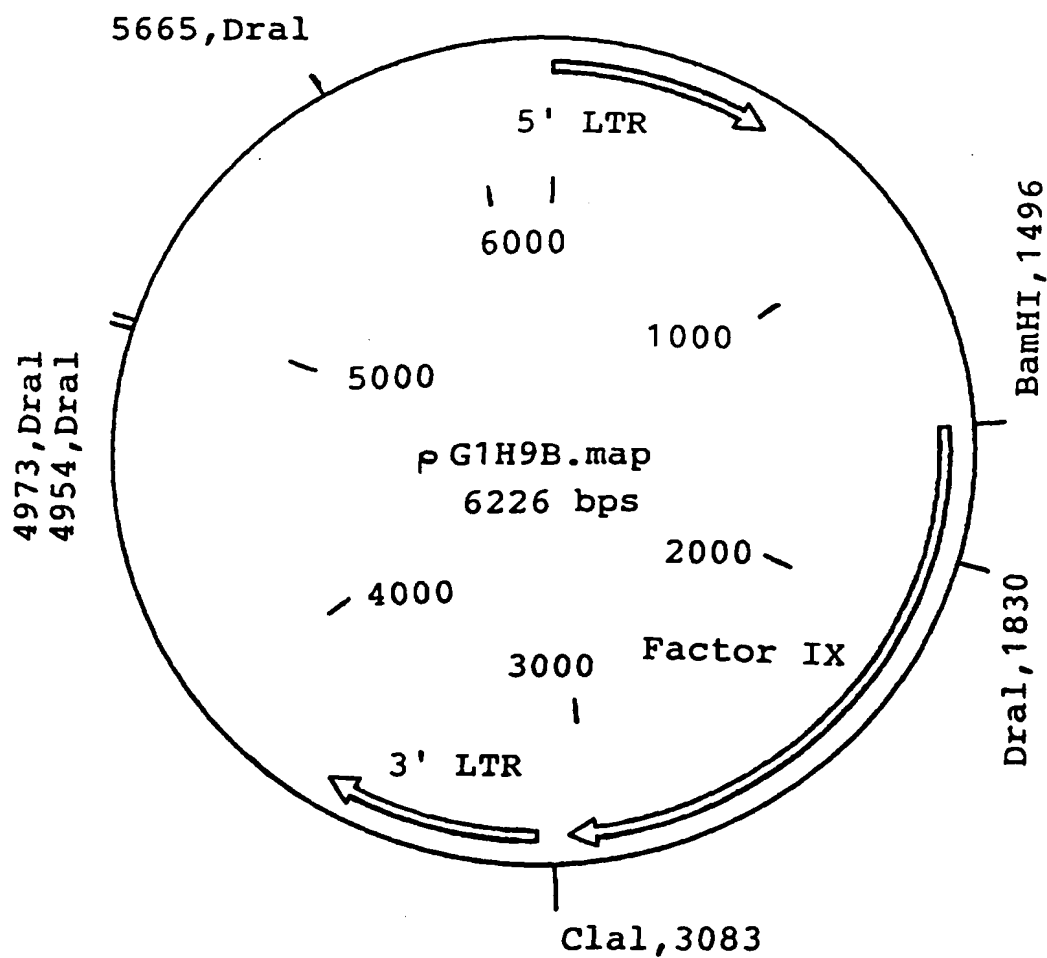
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FIG. 5



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FIG. 6



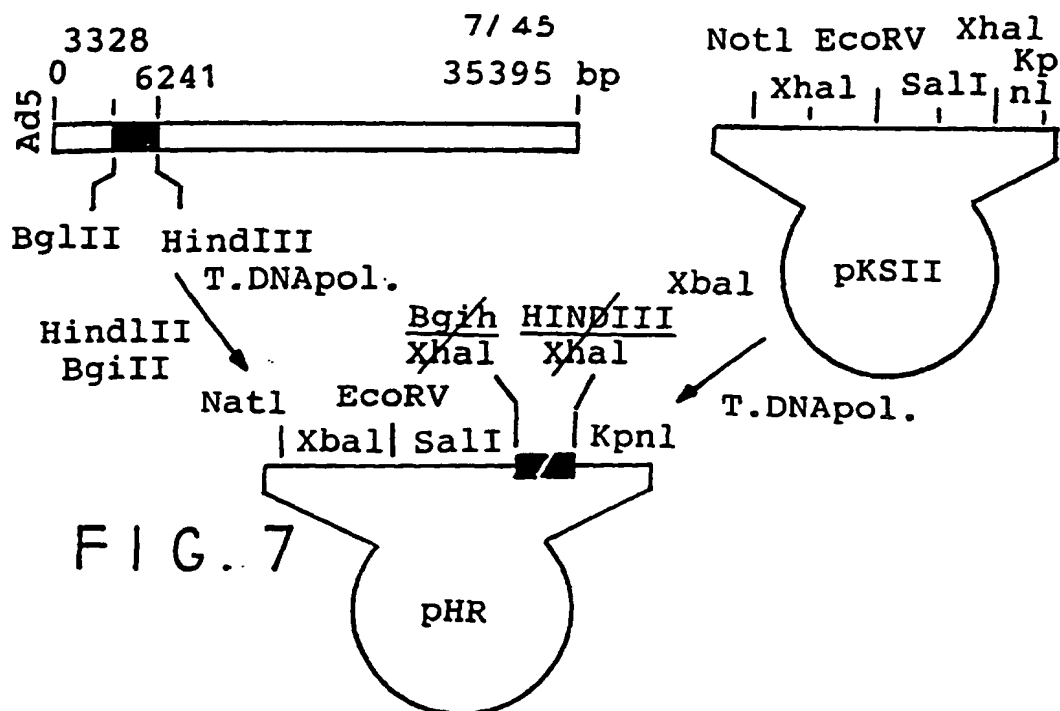


FIG. 7

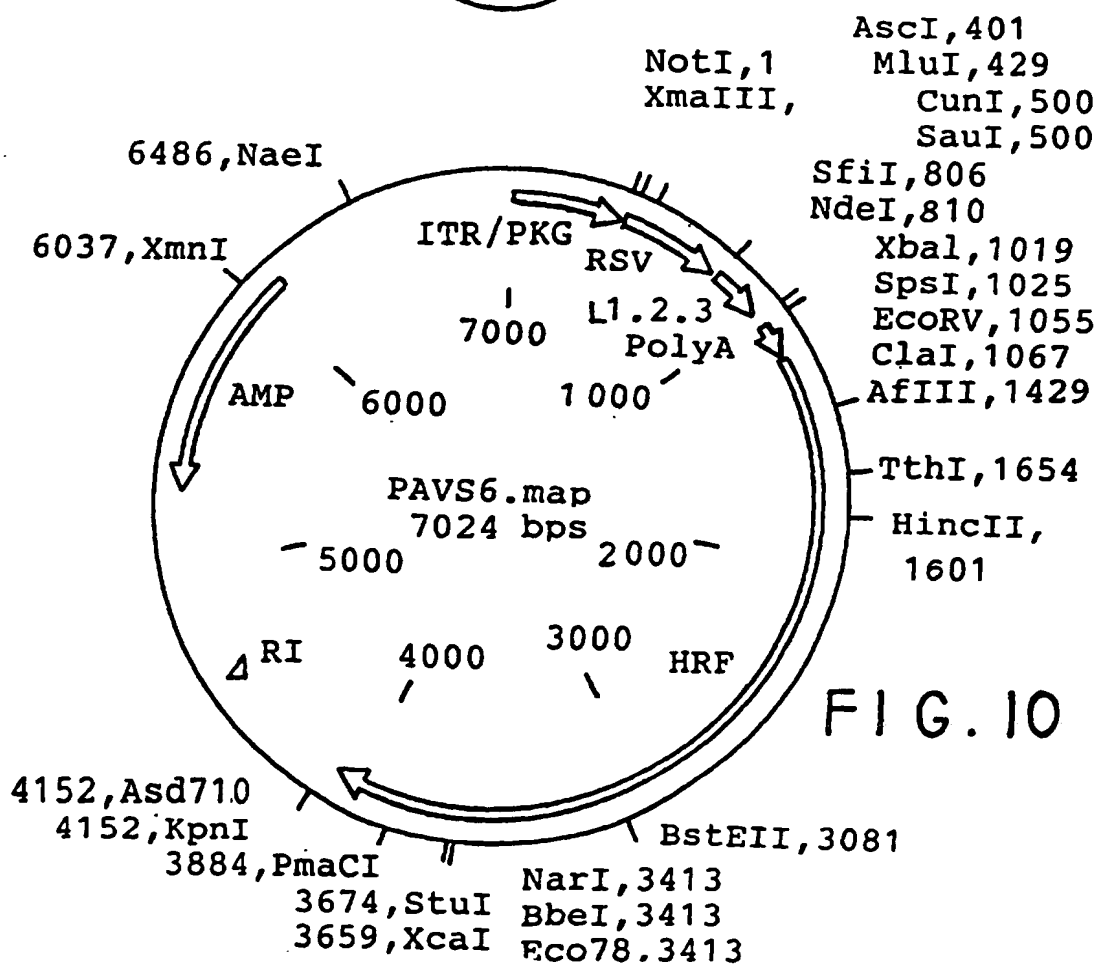


FIG. 10

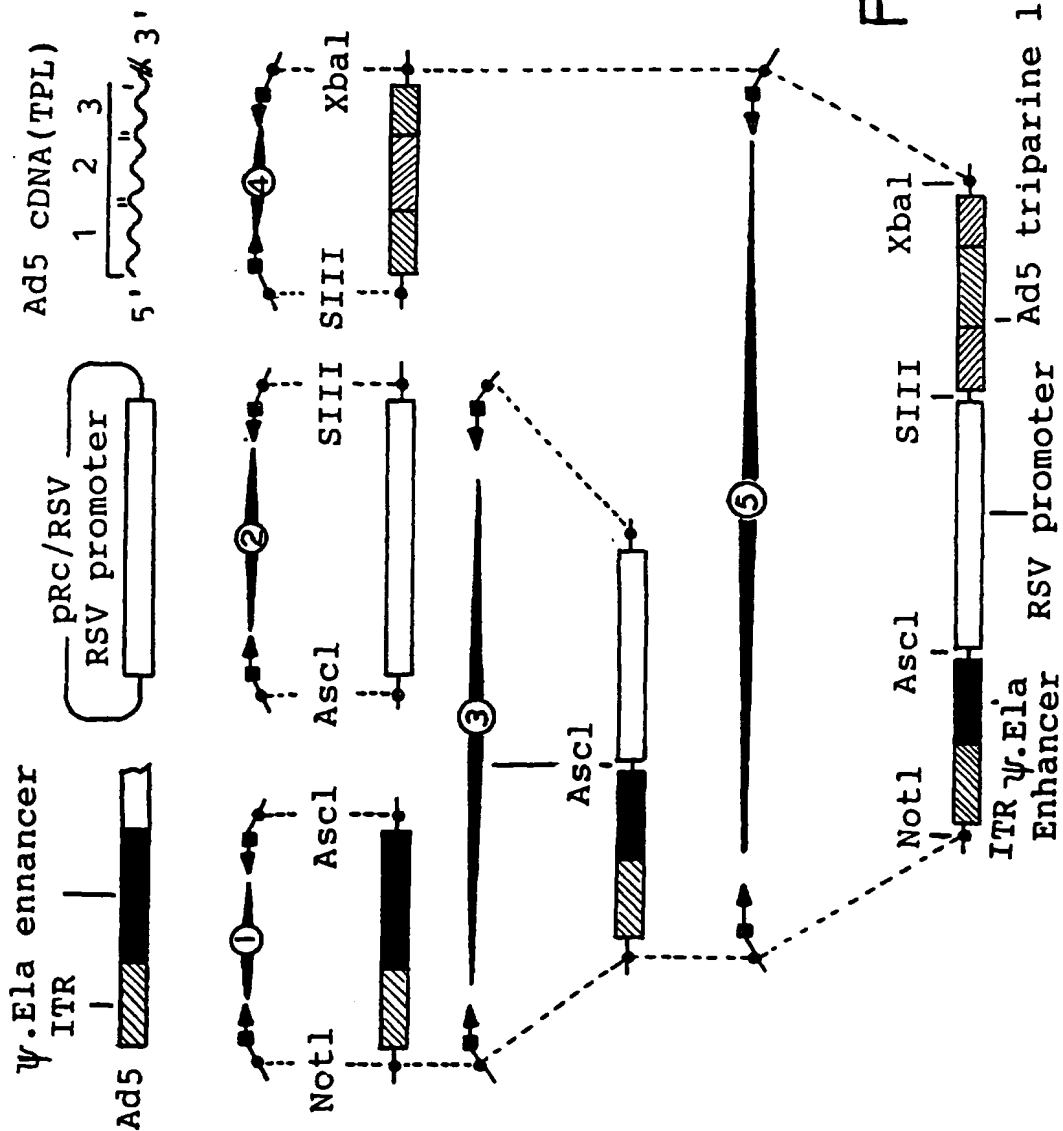
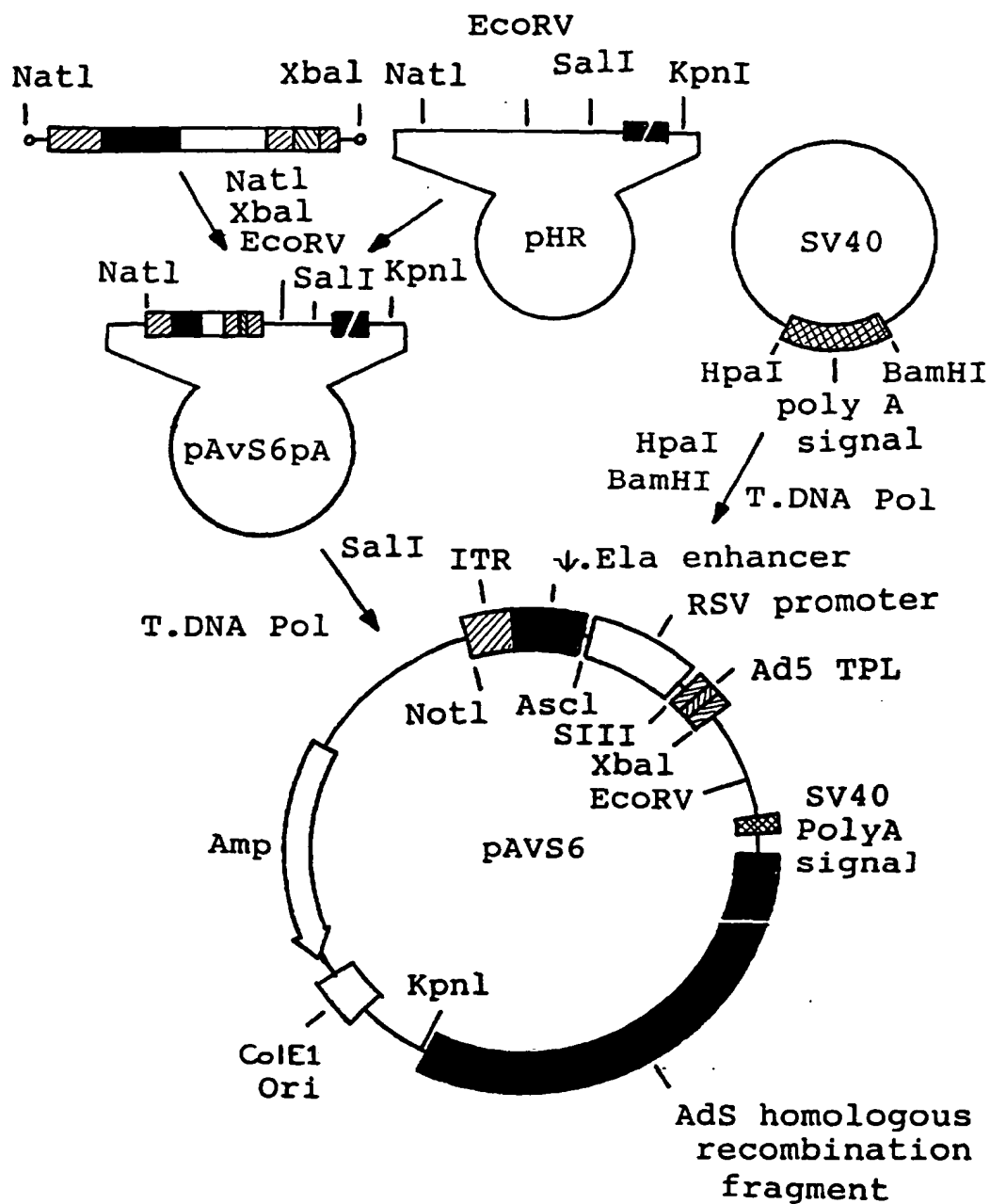


FIG. 8

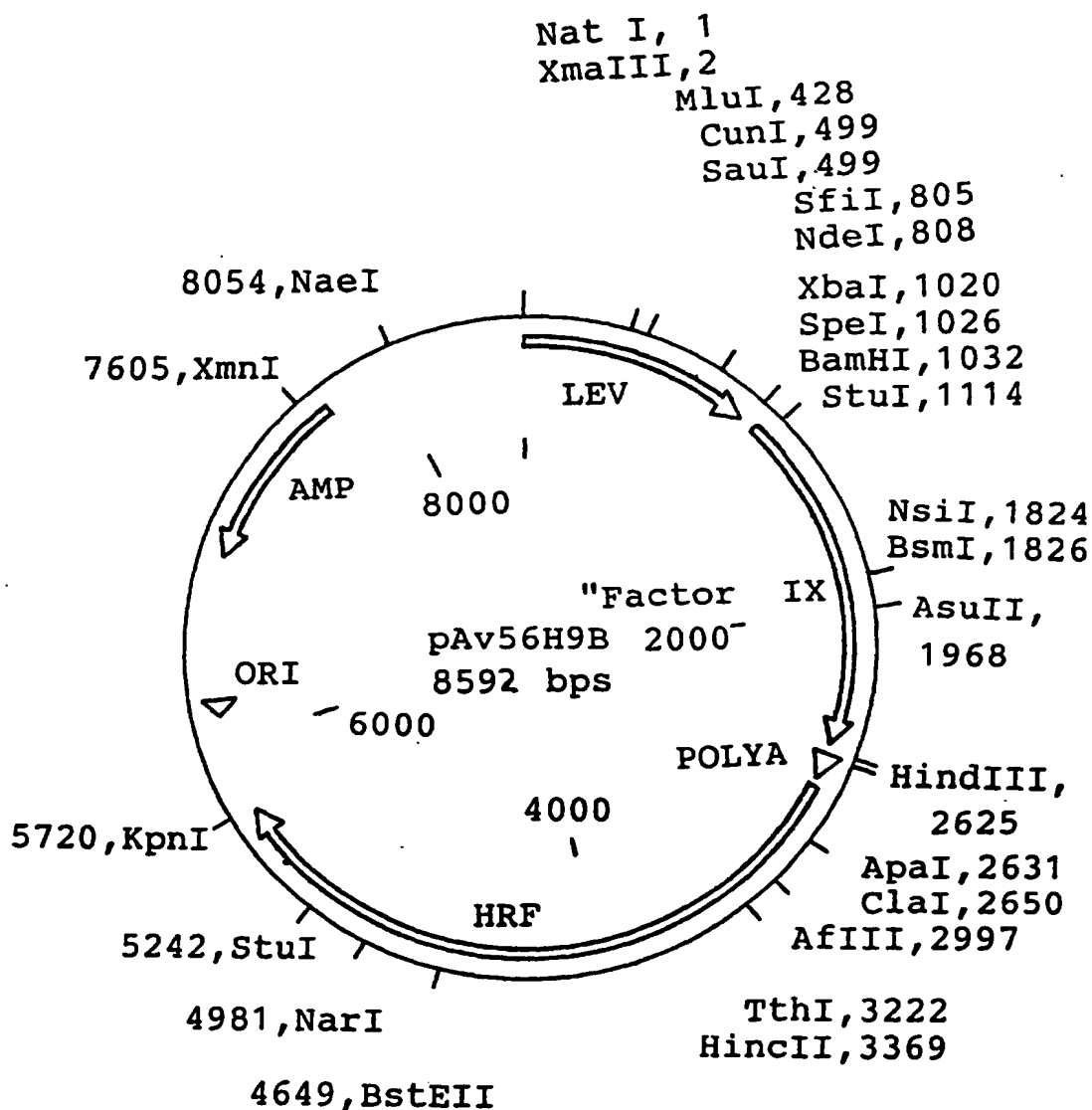
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FIG. 9



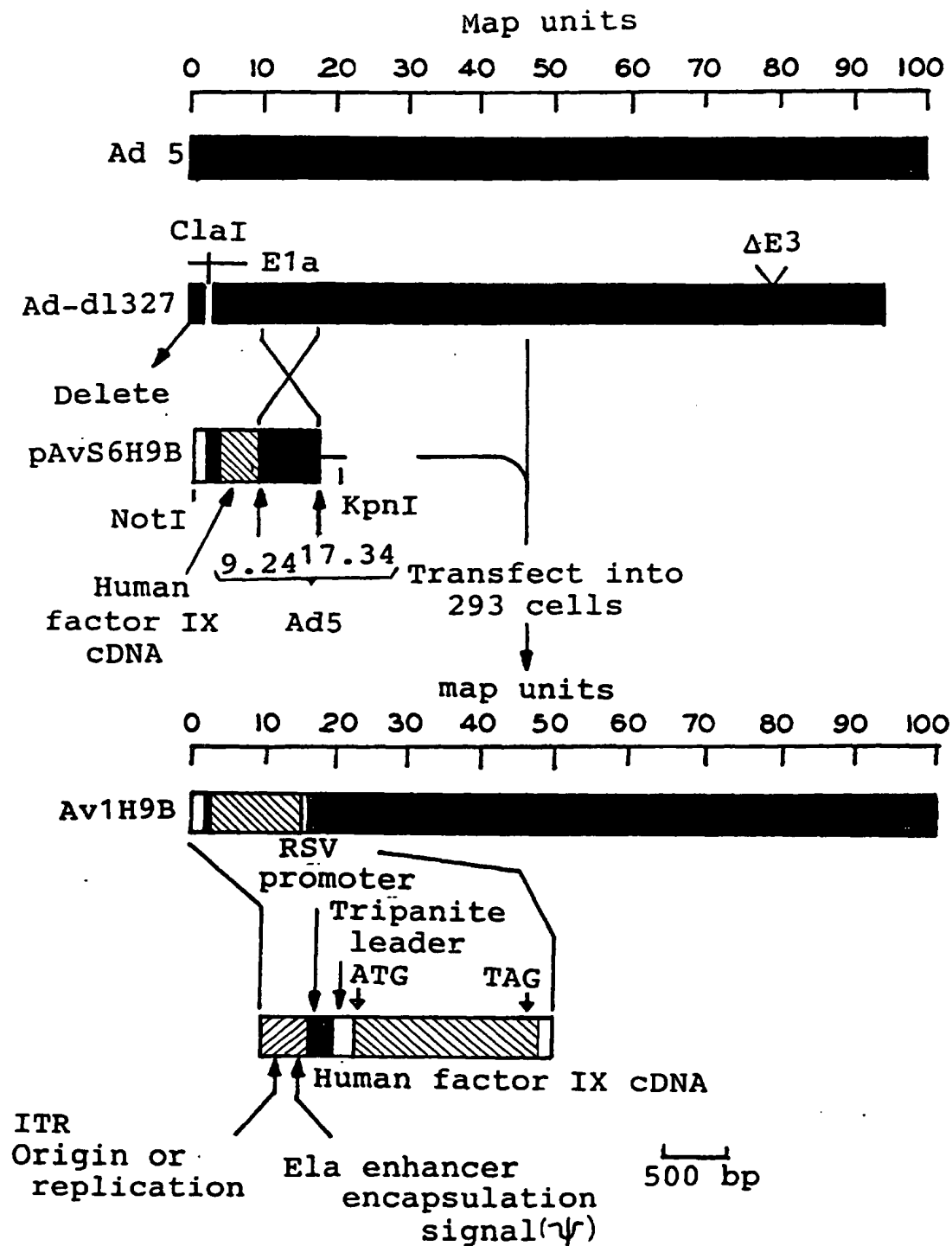
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FIG. 12

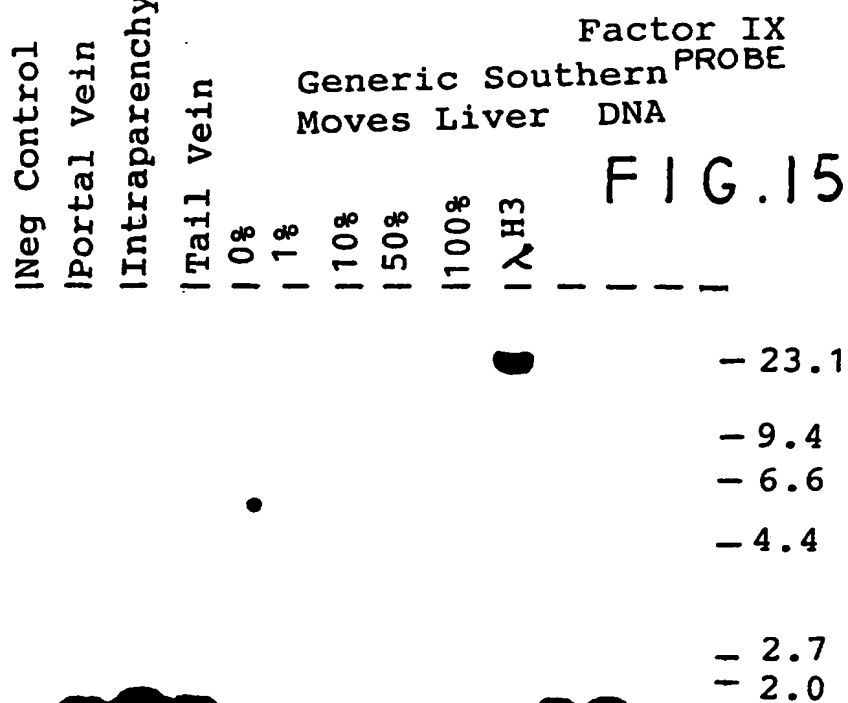
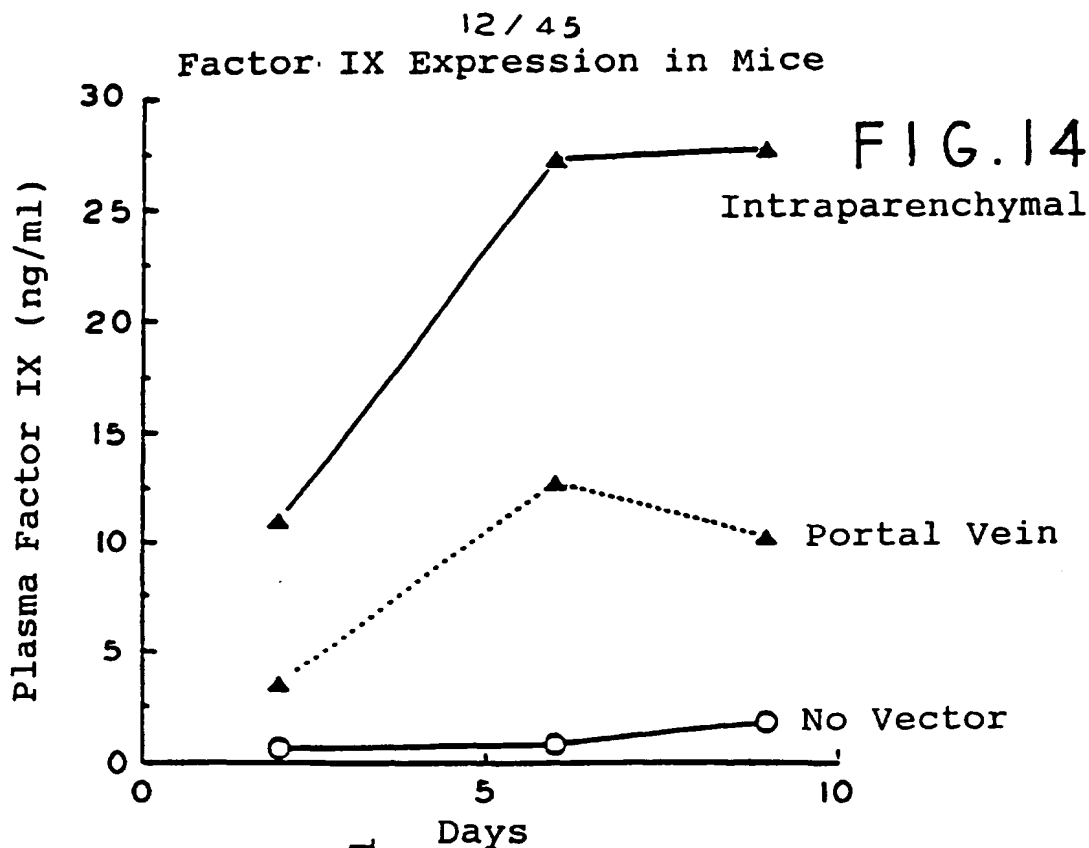


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FIG. 13

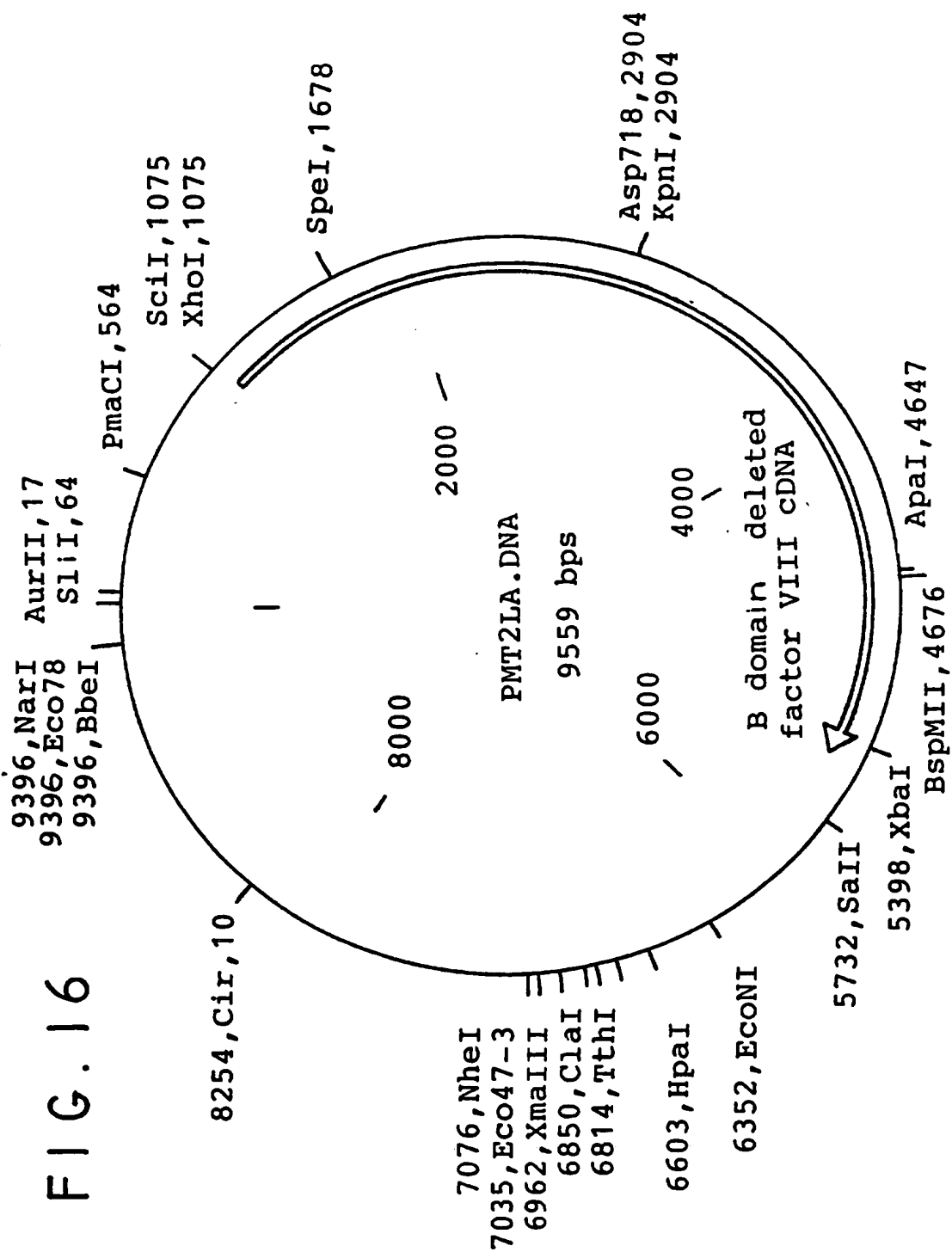


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## FIG. 17A

1 ATGCAAATAG AGCTCTCCAC CTGCTTCTTT CTGTGCCTTT TCGGATTCTG CTTTAGTGCC  
61 ACCAGAAGAT ACTACCTGGG TGCAGTGGAA CTGTGATGGG ACTATATGCA AGTGATCTC  
121 GGTGAGCTGC CTGTGGACGC AAGATTTCCT CCTAGAGTGC CAAAATCTTT TCCATTGAAC  
181 ACCTCAGTCG TGTACAAAAA GACTCTGTTT GTAGAATTCA CGGTTACCTT TTTCAACATC  
241 GCTAAGCCAA GGCCACCCCTG GATGGGTCG TAAGAACATG GCTTCCCCTA CCATCCAGGC TGAGGTTTAT  
301 GATACAGTGG TCATTACACT ACTGGAAAGC TTCTGAGGGA GCTGAATATG ATGATCAGAC CAGTCAAAGG  
361 GGTGTATCCT CTGTAATCCT TAAAGAACATG GCTTCCCCTA GCTGTCAGTCT TCATGCTGTC  
421 GAGAAAGAAG ATGATAAAGT GTCCCAATGGC CTCTGACCCA CTGTGCCCTTA CCTACTCATA TCTTTCTCAT  
481 AAGAGAAATG GTCCCAATGGC TAAAGACTT GAATTCAGGC TTGCACAAAT TTATACTACT ATGTAGAGAA  
541 GTGGACCTGG TAAAGACTT CCAAGGAAA GACACAGACC GCACTCAGAA ACAAAGAACT CCTTGATGCA GGATAGGGAT  
601 GGGAGTCTGG TAAAGACTT CCAAGGAAA GCACTCAGAA GCACTCAGAA TCAAGTCTATT GGATGTGAT TGGAA'TGGGC  
661 TTTGATGAAG GGAAGGTTG CTCGGGCCCTG TGATTGGATG CCACAGGAAA AATATTCCTC GAAGTCTACA CATTTCTTGT GAGGAACCAT  
721 GCTGCATCTG AAGTGCACTC CCAAGGAAA GCACTCAGAA GCACTCAGAA TCAAGTCTATT GGATGTGAT TGGAA'TGGGC  
781 CTGCCAGGTC TGATTGGATG CCACAGGAAA AATATTCCTC GAAGTCTACA CATTTCTTGT GAGGAACCAT  
841 ACCACTCCTG AAGTGCACTC CCAAGGAAA GCACTCAGAA GCACTCAGAA TCAAGTCTATT GGATGTGAT TGGAA'TGGGC  
901 CGCCAGGCGT CCTTGGAAAT CTGCGCCAATA ACTTTCCTTA ATCTCTTCCC ACCAACATGA TGGCATGGAA  
961 GACCTTGGAC AGTTTCTACT GTTTTGTCTAT CTGTCCTTCC ACTTTCCTTA ATCTCTTCCC ACCAACATGA TGGCATGGAA  
1021 GCTTATGTCA AAGTAGACAG CTGTCCAGAG TGATCTTACT GATCTCGAAA TACGAATGAA AATAATGAA  
1081 GAAGCGGAAG ACTATGATGA TATCCAAATT CGCTCAGTTG CCAAGAAGCA TCCATAAACT  
1141 GATGACAACT CTCTTCCCTT TGAAGAGGAG GACTGGGACT ATGCTCCCTT AGTCCCTCGCC  
1201 TGGTGACATT ACATTGCTGC TGAAGAGGAG GACTGGGACT ATGCTCCCTT AGTCCCTCGCC  
1261 CCCGATGACA GAAGTTATAA AGTCAATAT TTGAACAATG GCCCTCAGCG GATGGGTAGG  
1321 AAGTACAAA AAGTCCGATT PATGGCATAC ACAGATGAAA CCTTTAAGAC TCGTGAAGCT  
1381 ATTCAGCATG AATCAGGAAT CTTGGGACCT TTACTTTATG GGAAGTTGG AGACACACTG  
1441 TTGATTATAT TTAAGAAATCA AGCAAGCAGA CCATATAACA TCTACCCCTCA CGGAATCACT  
1501 GATGTCCGTC CTTTGTATTC AAGGAGATTA CCAAAAGGTG TAAACATTT GAAGGATTTT

MATCH WITH FIG. 17B

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## FIG. 17B

MATCH WITH FIG. 17A

1561	CCAATTCTGC	CAGGAGAAAT	ATTCÀAATAT	AAATGGACAG	TGACTGTAGA	AGATGGGCCA
1621	ACTAAATCAG	ATCCTCGGTG	CCTGACCCCC	TATTACTCTA	GTTCGTTAA	TATGGAGAGA
1681	GATCTAGCTT	CAGGACTCAT	TGGCCCTCTC	CTCATCTGCT	ACAAAGAAAT	TGTAGATCAA
1741	AGAGGAAACC	AGATAATGTC	AGACAAGAGG	AATGTCATCC	TGTTTTCTGT	ATTTGATGAG
1801	AACCGAAGCT	GGTACCTCAC	AGAGAATATA	CAACGCTTTC	TCCCCAATCC	AGCTGGAGTG
1861	CAGCTTGAGG	ATCCAGAGTT	CCAAGCCCTC	AACATCATGC	ACAGCATCAA	TGGCTATGTT
1921	TTTGATAGTT	TGCAGTTGTC	AGTTTGTTC	CATCACCTGG	CATACTGGTA	CATTCTAAGC
1981	ATTGGAGCAC	AGACTGACTT	CCTTCTCTGC	TTCTTCTCTG	GATATACCTT	CAAAACACAAA
2041	ATGGTCTATG	AAGACACACT	CACCCATATC	CCATTCTCAG	GAGAAACTGT	CTTCATGTCG
2101	ATGGAAAACC	CAGGTCATAG	GATTCTGGGG	TGCCACAAC	CAGACTTTCG	GAACAGAGGC
2161	ATGACCCGCT	TACTGAAGGT	TTCTAGTTGT	GACAAGAACA	CTGGTGATTA	TTACGAGGAC
2221	AGTTATGAAG	ATATTTCAGC	ATACTTGCTG	AGTAAAAACA	ATGCCATTGA	ACCAAGAAGC
2281	TTCTCCCCAGA	ATTCAAGAGA	CCCTAGCCT	AGGCAAAAGC	AATTTAATGC	CACCCACCCA
2341	GTCTTGAAAC	GCCATCAACG	GGAATAAAT	CGTACTACTC	TTCAGTCAGA	TCAAGAGGAA
2401	ATTGACTATG	ATGATACCAT	ATCAGTTGAA	ATGAAGAAGG	AAGATTTTGA	CATTATGAT
2461	GAGGATGAAA	ATCAGAGCCC	CCGCAGCTTT	CAAAAGAAA	CACGACACTA	TTTTATTGCT
2521	GCAGTGGAGA	GGCTCTGGGA	TTATGGGATG	ACTAGCTCCC	CACATGTTGT	AAGAAAGAGG
2581	GCTCAGAGTG	GCAGTGTCCC	TCAGTTC AAG	AAAGTTGTTT	TCCAGGAATT	TACTGATGGC
2641	TCCTTTACTC	AGCCCTTATA	CCGTGGAGAA	CTAAATGAAC	ATTTGGGACT	CCTGGGGCCA
2701	TATATAAGAG	CAGAAAGTTGA	AGATAATATC	ATGGTAACTT	TCAGAAATCA	GGCTCTTCGT
2761	CCCTATTCCCT	TCTATTCTAG	CCTTATTCTT	TATGAGGAAG	ATCAGAGGCA	AGGAGCAGAA
2821	CCTAGAAAAA	ACTTTGTCAA	GCCTAATGAA	ACCAAAACTT	ACTTTTGGA	AGTGCAACAT
2881	CATATGGCAC	CCACTAAAGA	TGAGTTTGAC	TGCAAAAGCCT	GGGCTTATTT	CTCTGATGTT
2941	GACCTGGAAA	AAGATGTGCA	CTCAGGCCCTG	ATTGGACCCC	TTCTGTGCTG	CCACACTAAC
3001	ACACTGAACC	CTGCTCATGG	GAGACAAAGT	ACAGTACAGG	AATTTGCTCT	GTTTTTCACC
3061	ATCTTTGATG	AGACCAAAAG	CTGGTACTTC	ACTGAAAATA	TGGAAGAGAA	CTGCAGGGCT

MATCH WITH FIG. 17C

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## FIG. 17C

MATCH WITH FIG. 17B

3121	CCCTGCAATA	TCCAGATGGA	AGATCCCACT	TTTAAAGAGA	ATTATCGCTT	CCATGCAATC
3181	AATGGCTACA	TAATGGATAC	ACTACCTGGC	TTAGTAATGG	CTCAGGATCA	AAGGATTCGA
3241	TGGTATCTGC	CTAGCATGGG	GAGCAATGAA	AACATCCATT	CTATTTCATT	CAGTGGACAT
3301	GTGTTCACTG	TACGAAAAAA	AGAGGAGTAT	AAAATGGCAC	TGTACAATCT	CTATCCAGGT
3361	GTTTTTGAGA	CAGTGGAAAT	GTACCATCC	AAAGCTGGAA	TTTGGCGGGT	GGAAATGCCTT
3421	ATTGGCGAGC	ATCTACATGC	TGGGATGAGC	ACACTTTTTC	TGGTGTACAG	CAATAAGTGT
3481	CAGACTCCCC	TGGGAATGGC	TTCTGGACAC	ATTAGAGATT	TTCAGATTAC	AGCTTCAGGA
3541	CAATATGGAC	AGTGGGCCCC	AAAGCTGGCC	AGACTTCATT	ATTCCGGATC	AATCAATGCC
3601	TGGAGCACCA	AGGAGCCCCT	TTCTTGGATC	AAGGTGGATC	TGTTGGCAC	AATGATTATT
3661	CACGGCATCA	AGACCCAGGG	TGCCCGTCAG	AAGTCTCCA	GCCTCTACAT	CTCTCAGTTT
3721	ATCATCATGT	ATAGTCTTGA	TGGGAAGAAG	TGGCAGACTT	ATCGAGGAAA	TTCCACTGGA
3781	ACCTTAATGG	TCTTCTTTGG	CAATGTGGAT	TCATCTGGGA	TAAACACAAA	TATTTTAAAC
3841	CCTCCAATTA	TTGCTCGATA	CATCCGTTTG	CACCCAACCT	ATTATAGCAT	TCGCAGCACT
3901	CTTCGCATGG	AGTTGATGGG	CTGTGATTTA	AATAGTTGCA	GCATGCCATT	GGGAA'GGAG
3961	AGTAAAGCAA	TATCAGATGC	ACAGATTACT	GCTTCATCCT	ACTTTACCAA	TATGTTGCC
4021	ACCTGGTCTC	CTTCAAAAGC	TCGACTTCAC	CTCCAAGGGA	GGAGTAATGC	CTGGAGACCT
4081	CAGGTGAATA	ATCCAAAAGA	GTGGCTGCAA	GTGGACTTCC	AGAAGACAA	GAAAGTCACA
4141	GGAGTAACTA	CTCAGGGAGT	AAAATCTCTG	CTTACCAGCA	TGTATGTGAA	GGAGTTCCTC
4201	ATCTCCAGCA	GTCAAGATGG	CCATCAGTGG	ACTTCTTTT	TTCAGAAATGG	CAAAGTAAAG
4261	GTTTTTCAGG	GAAATCAAGA	CTCCTTCACA	CCTGTGGTGA	ACTCTCTAGA	CCCACCGTTA
4321	CTGACTCGCT	ACCTTCGAAT	TCACCCCCAG	AGTTGGTGGC	ACCAGATTGC	CCTGAGGATG
4381	GAGGTTCTGG	GCTGCGAGGC	ACAGGACCTC	TACTGAGGGT	GGCCACTGCA	GCACCTGCCA
4441	CTGCCGTCAC	CTCTCCCTCC	TCAGCTCCAG	GGCAGTGTCC	CTCCCTGGCT	TGCCTTCTAC
4501	CTTTGTGCTA	AATCCTAGCA	GACACTGCCT	TGAAGCCTCC	TGAATTAACT	ATCATCAGTC
4561	CTGCATTCT	TTGGTGGGG	GCCAGGAGGG	TGCATCCCAAT	TTAACTTAAC	TCTTACCCTAT
4621	TTTCTGCAG					

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FIG. 18

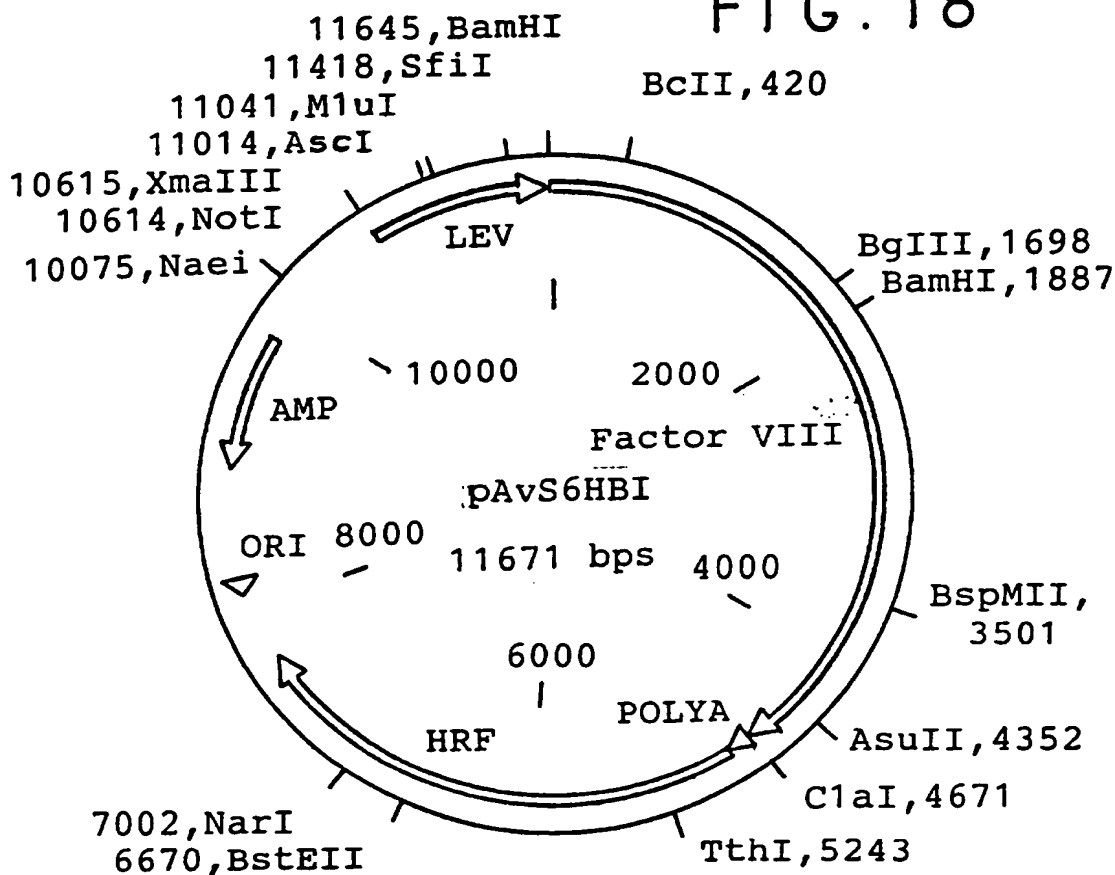
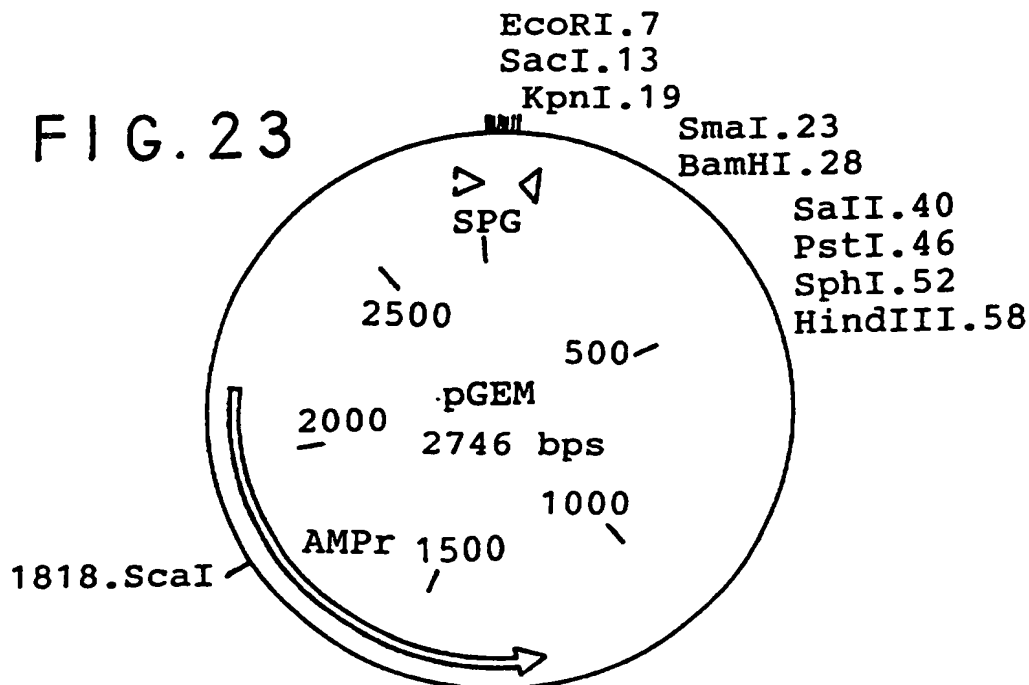


FIG. 23



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## FIG. 19

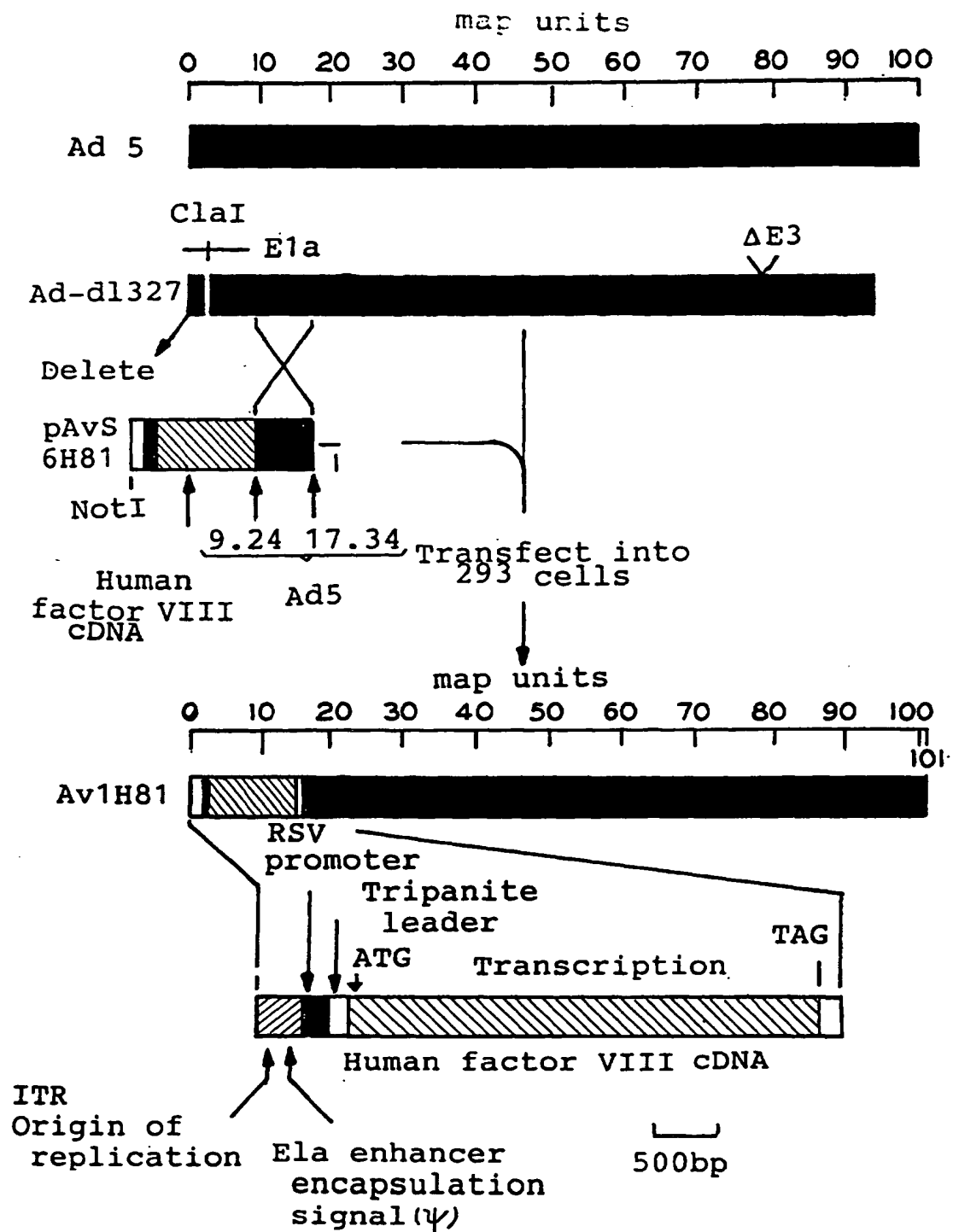


FIG. 20

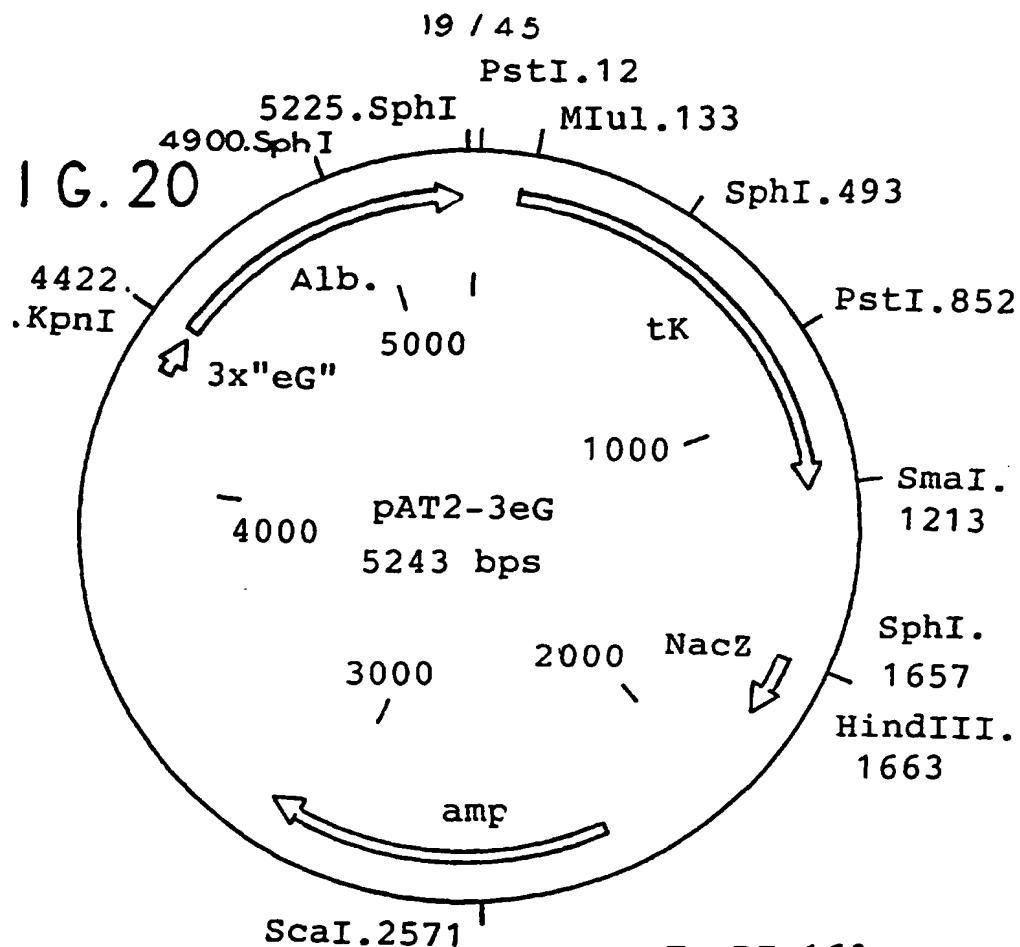
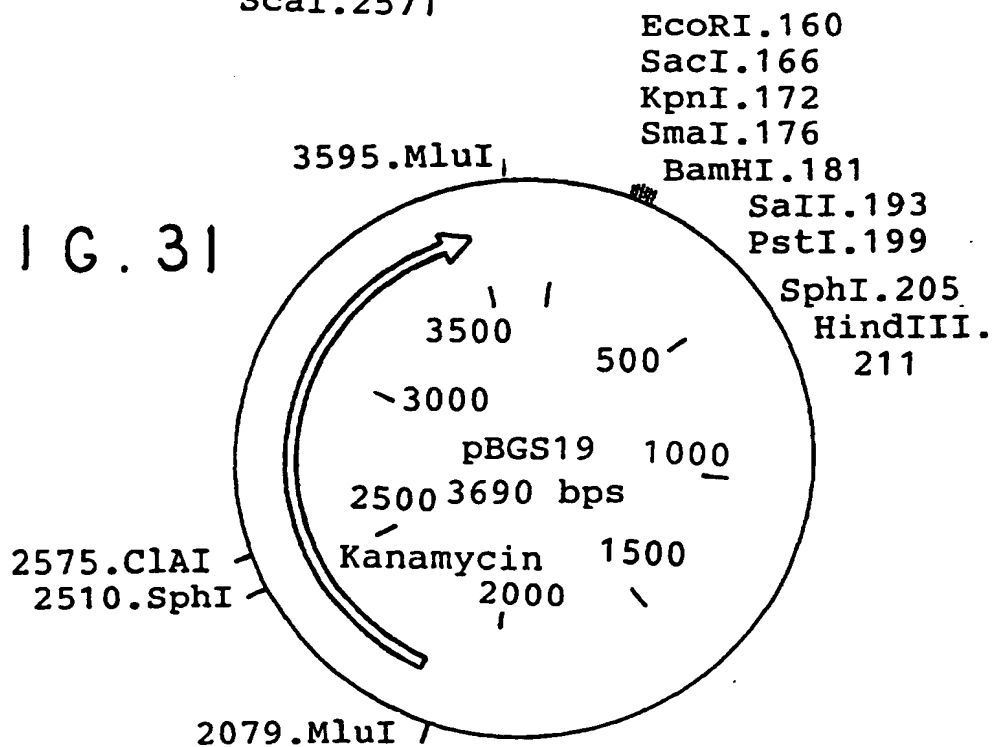


FIG. 31



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FIG. 21

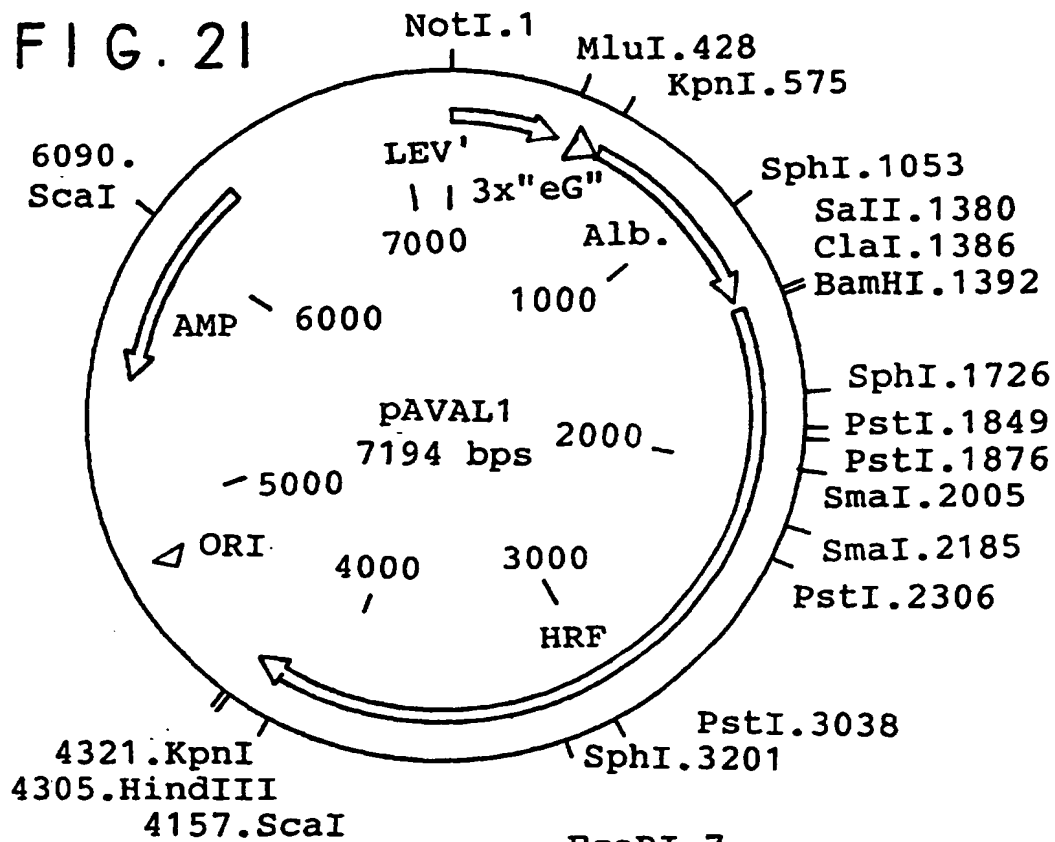


FIG. 22

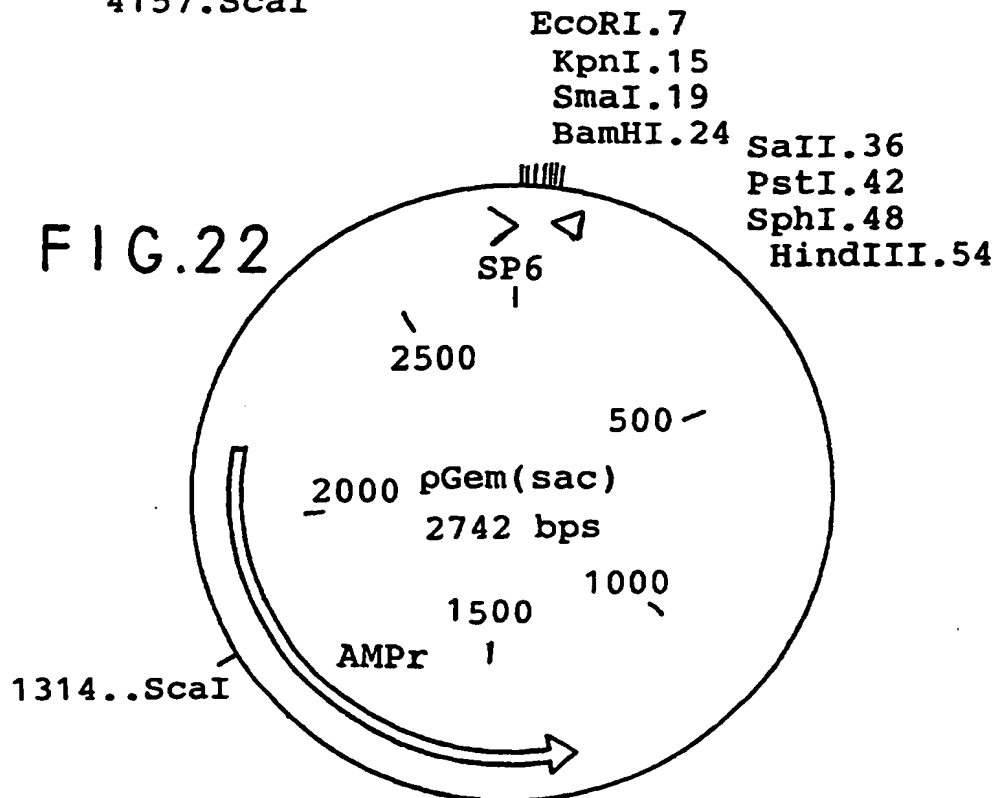




FIG. 24

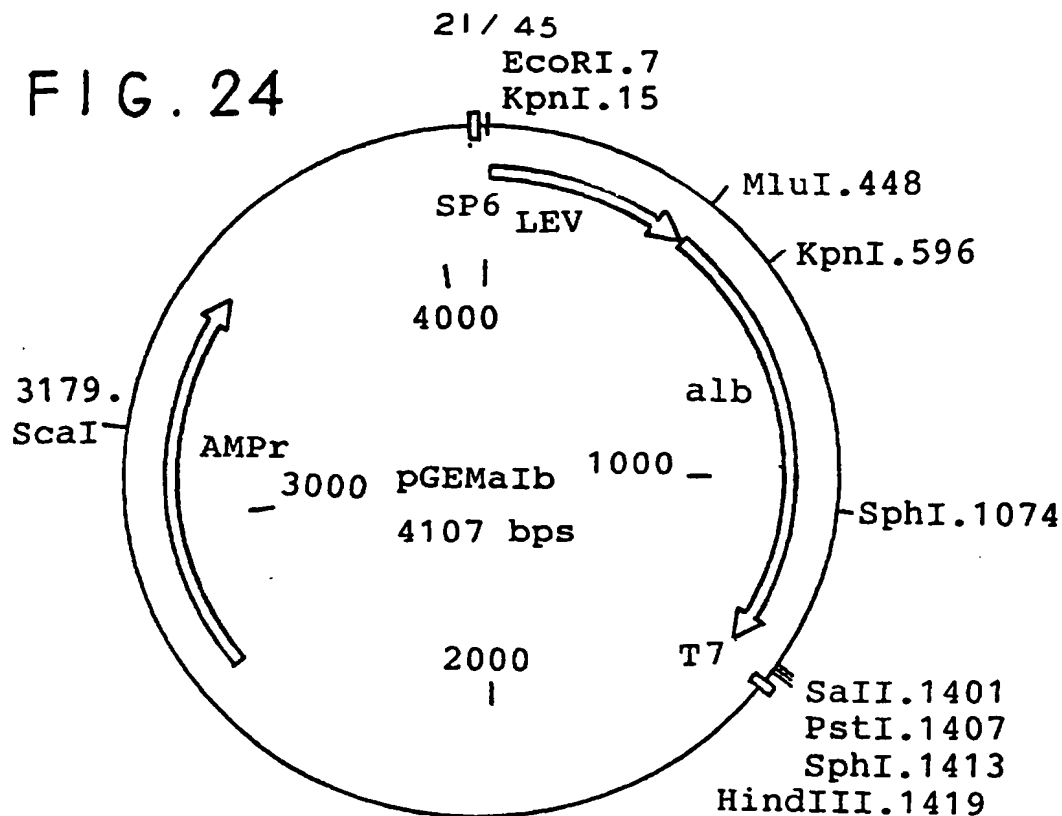


FIG. 25

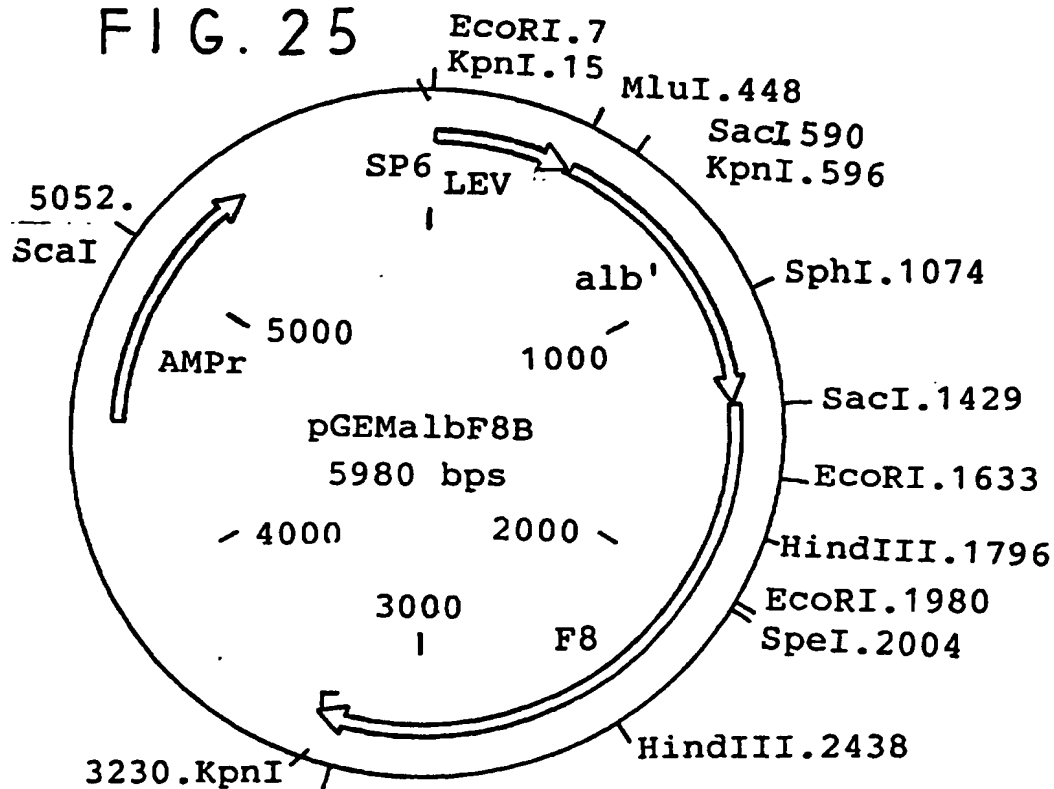


FIG. 26

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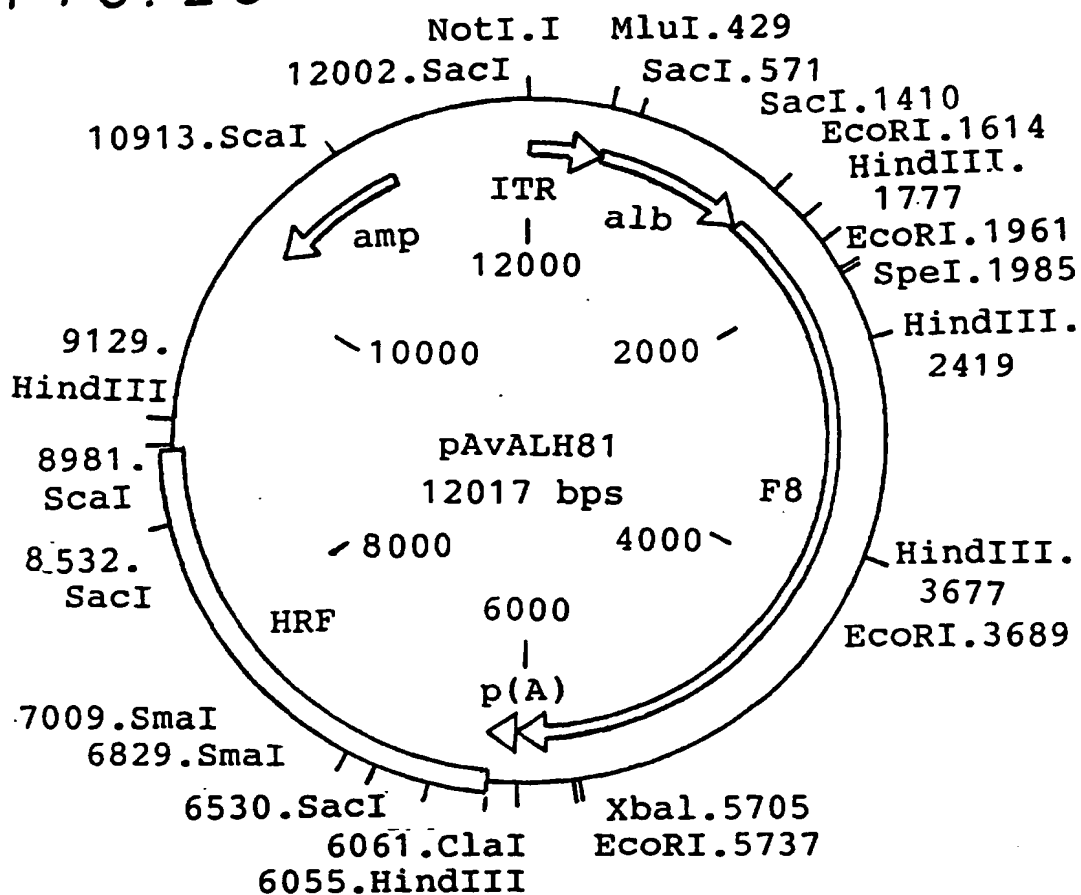
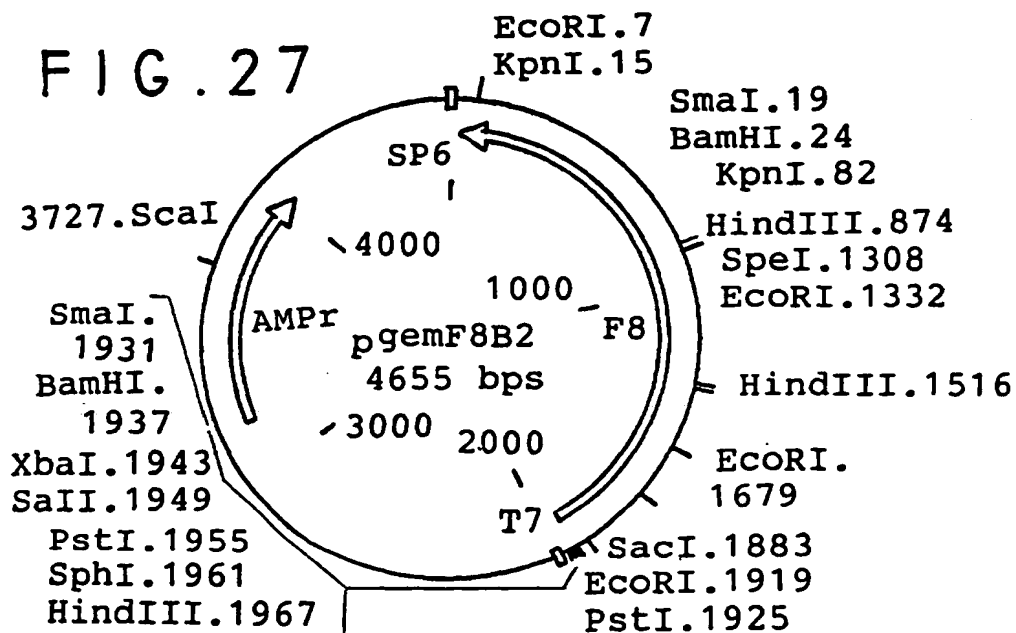


FIG. 27



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FIG. 28

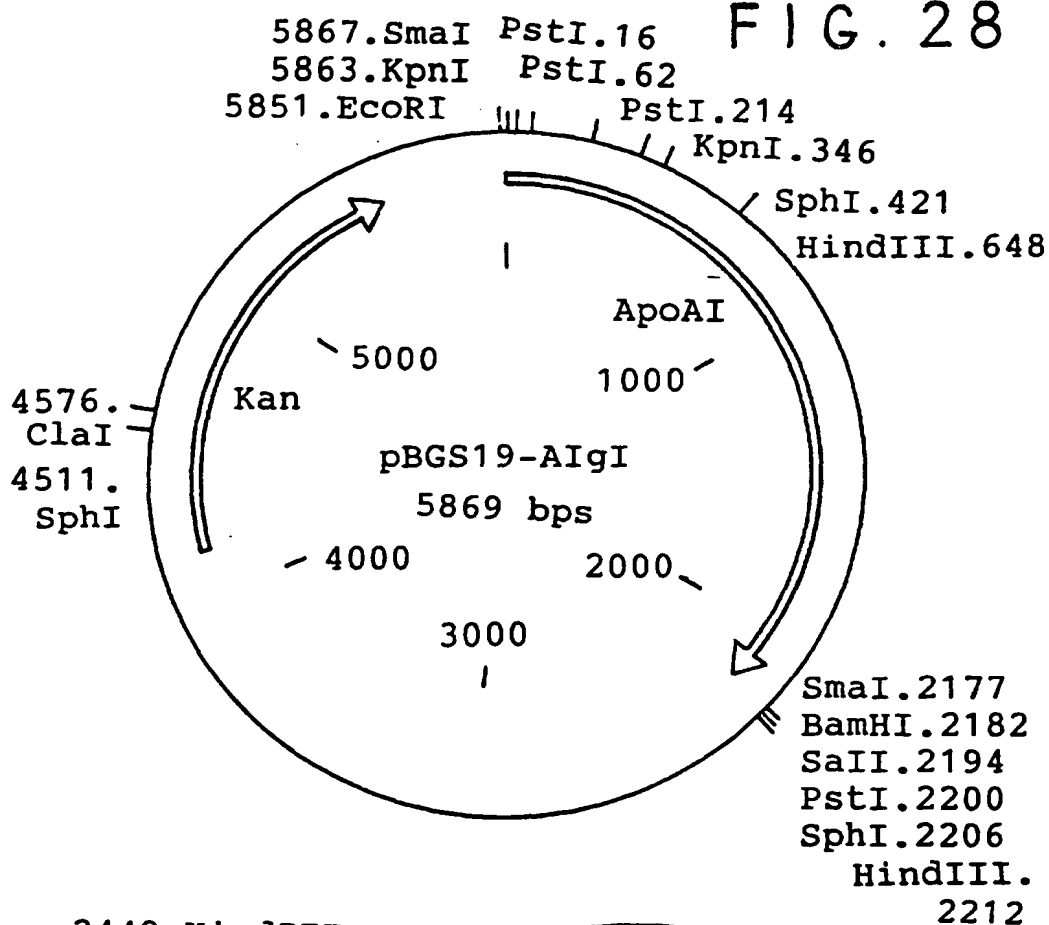
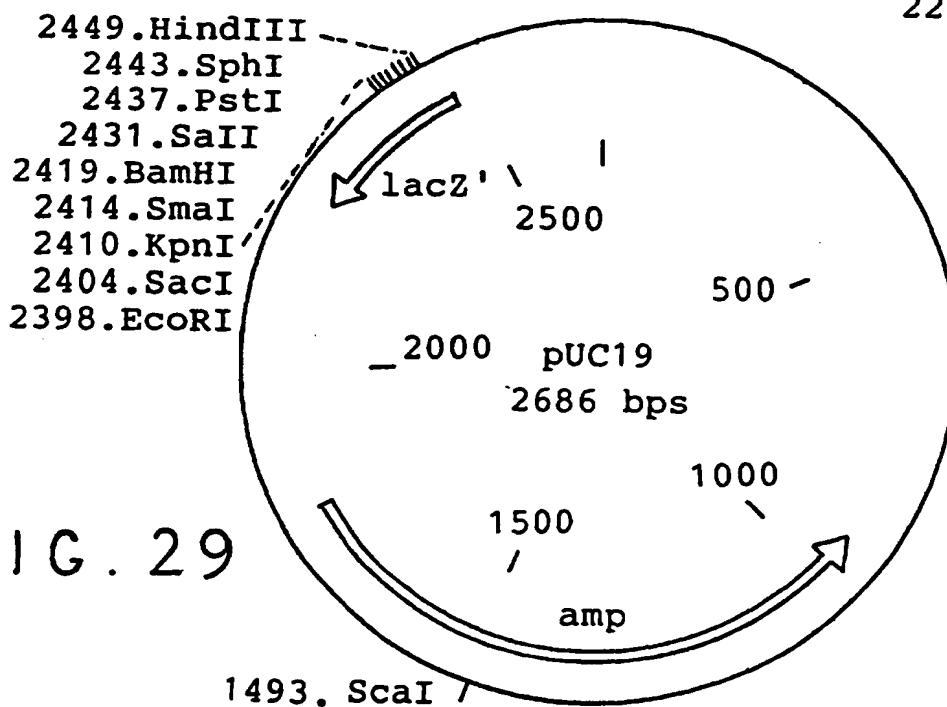


FIG. 29



SUBSTITUTE SHEET (RULE 26)

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FIG. 30

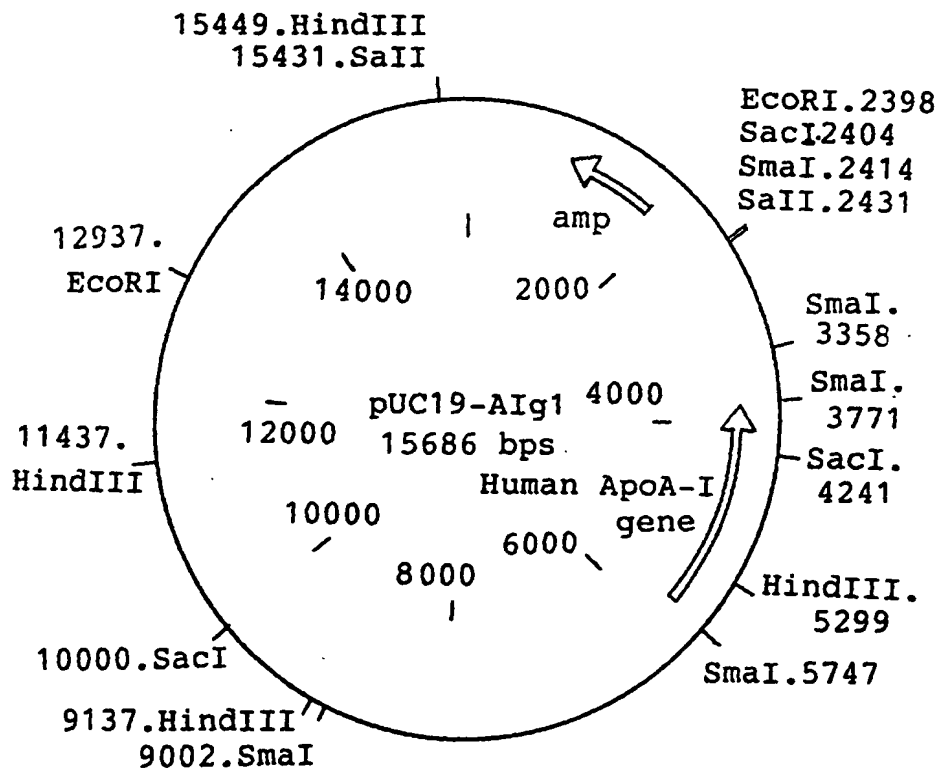


FIG. 33

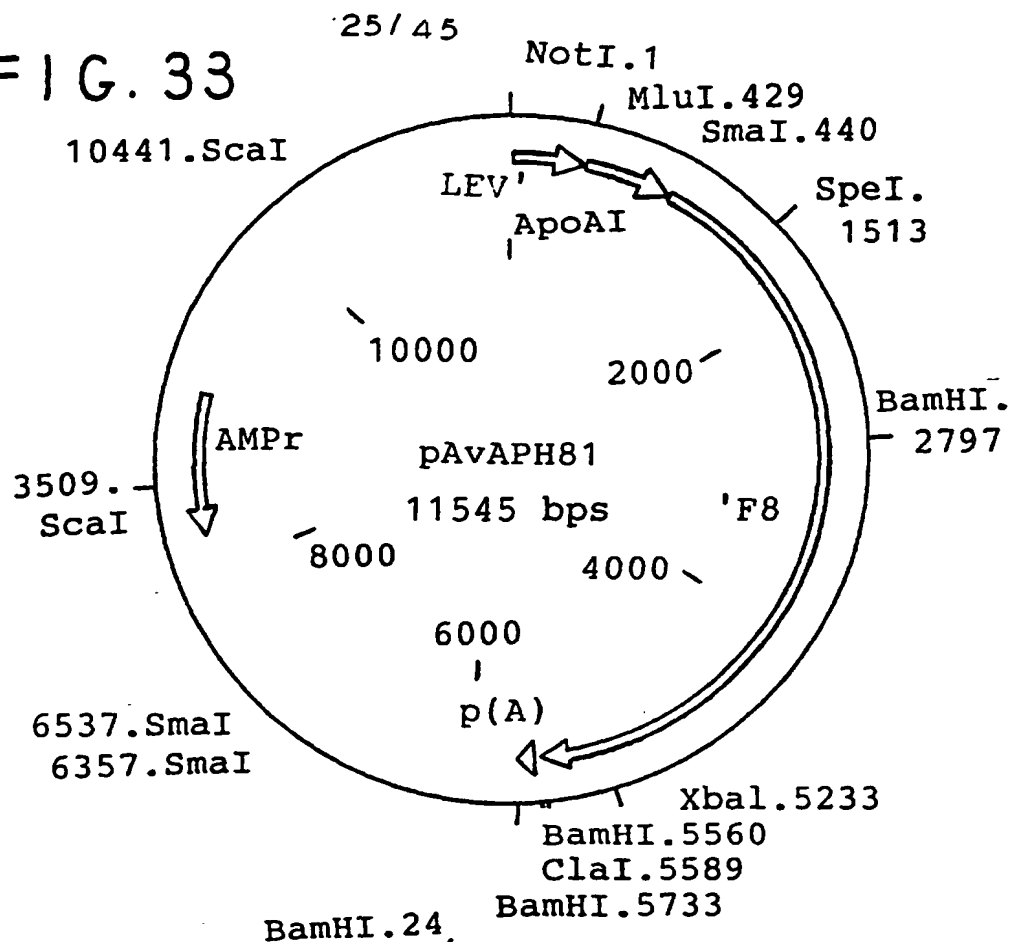


FIG. 32

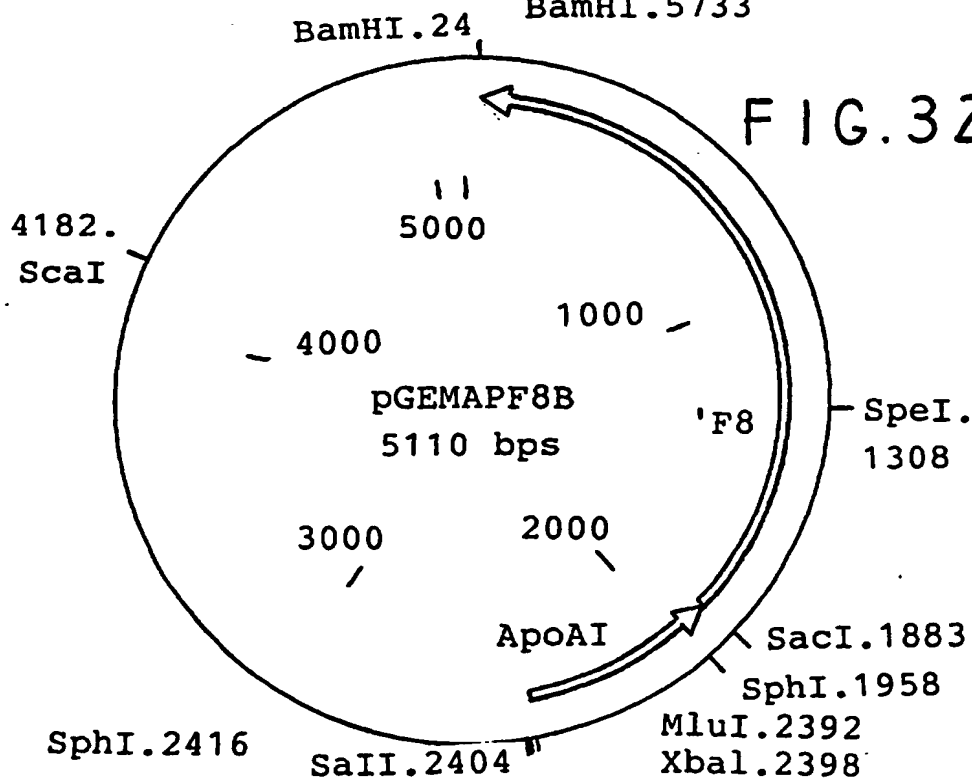


FIG. 34

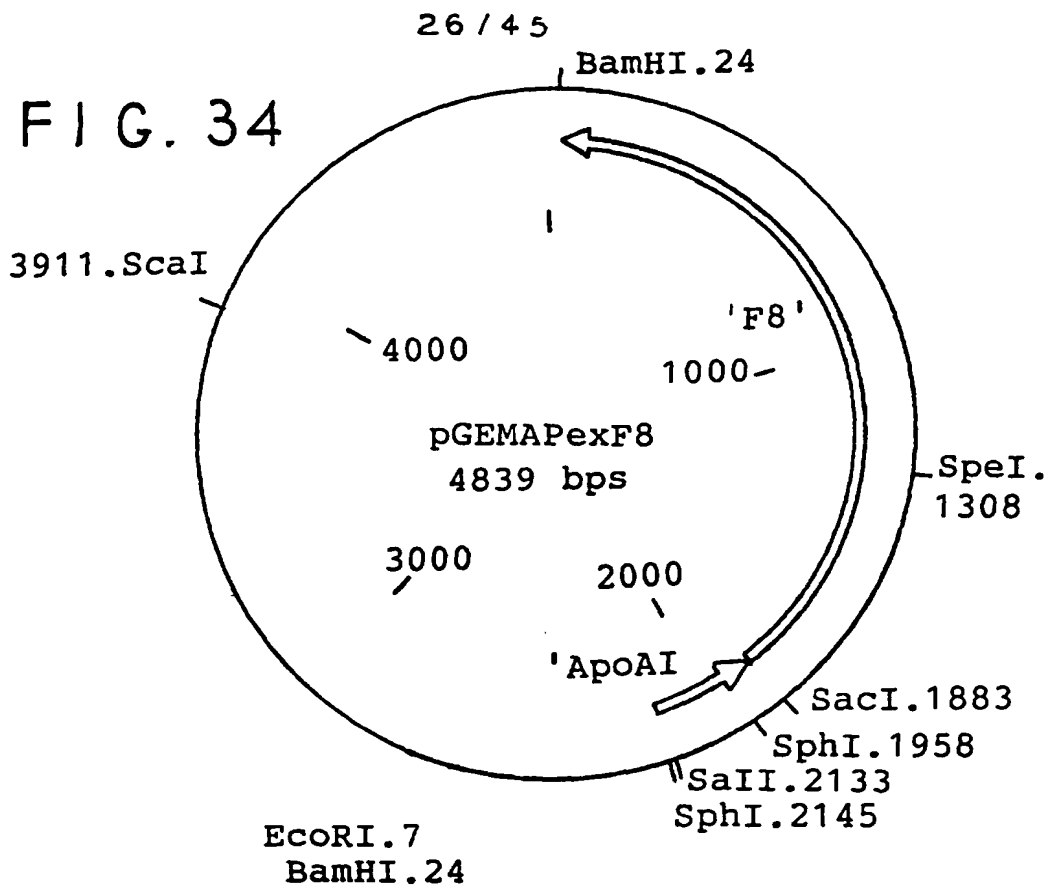
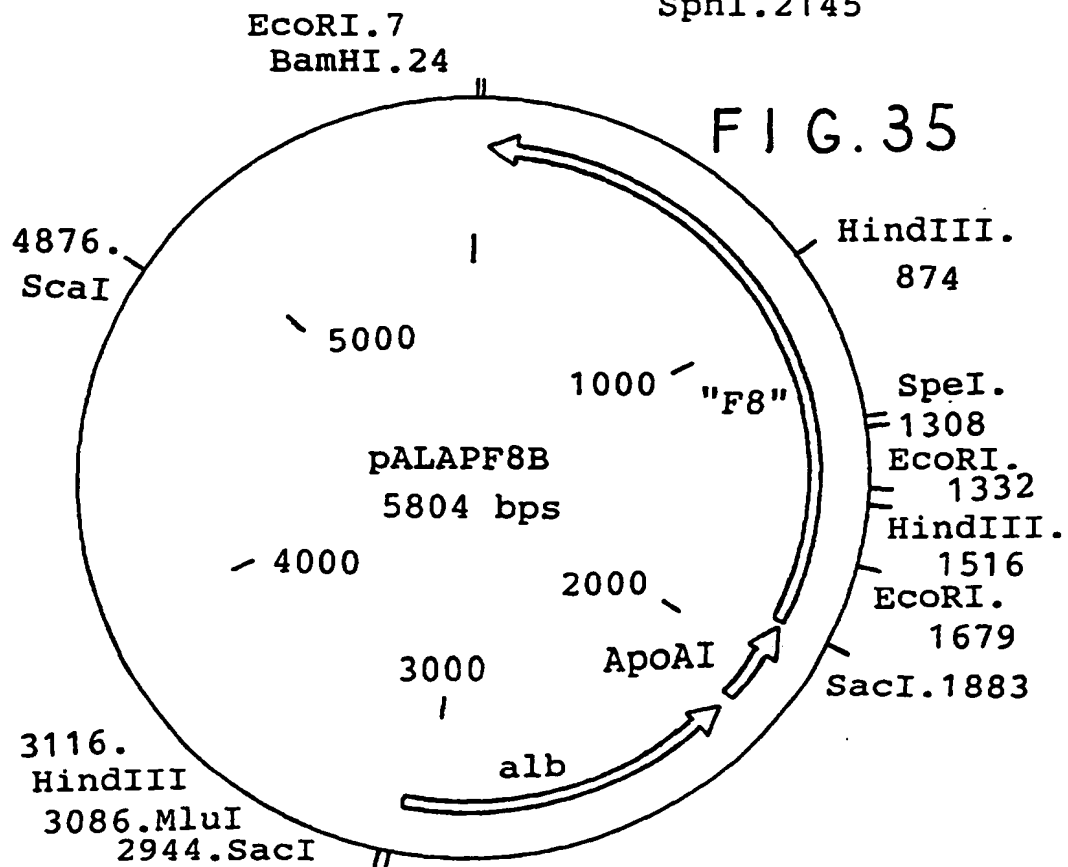


FIG. 35



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FIG. 36

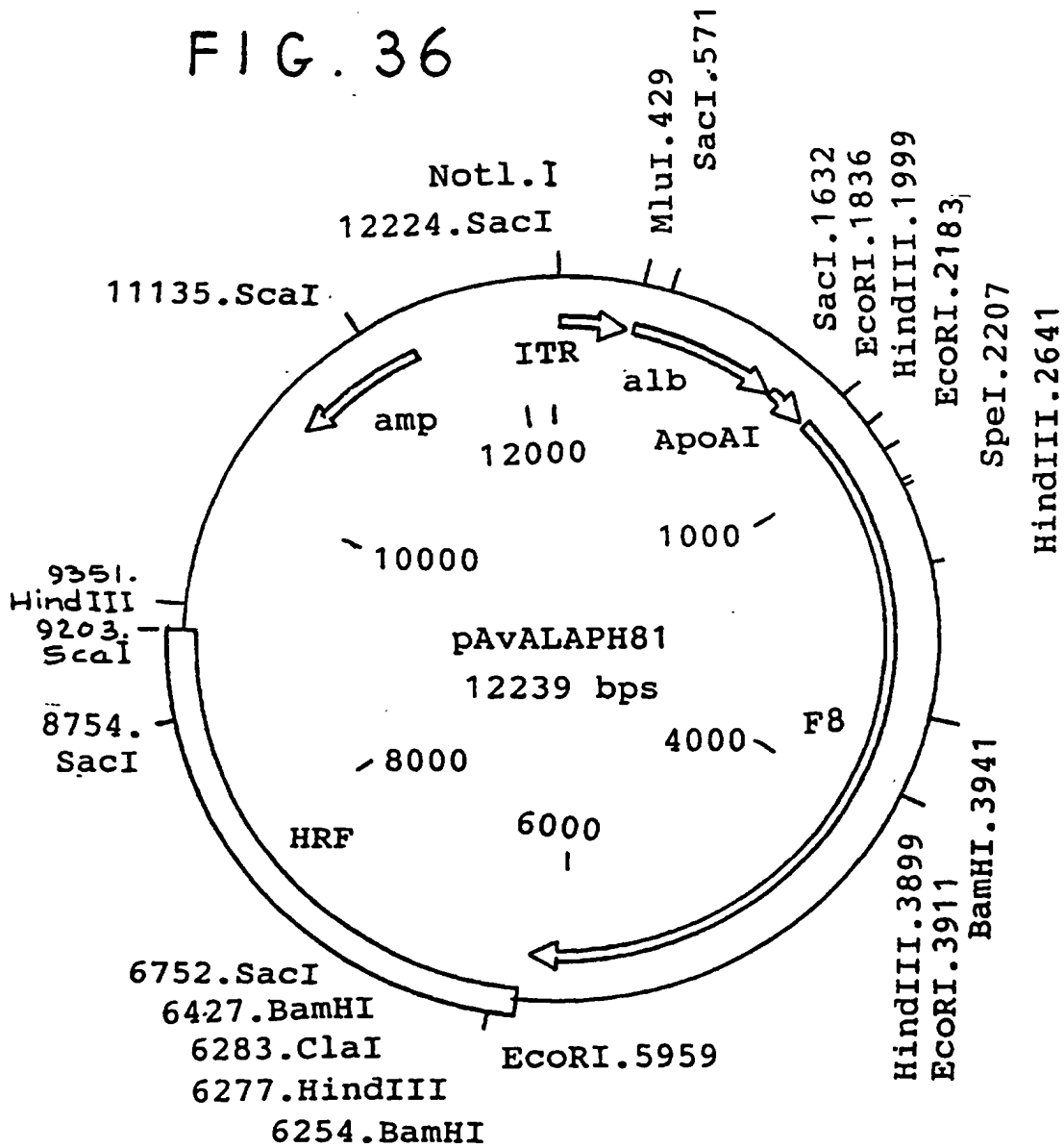
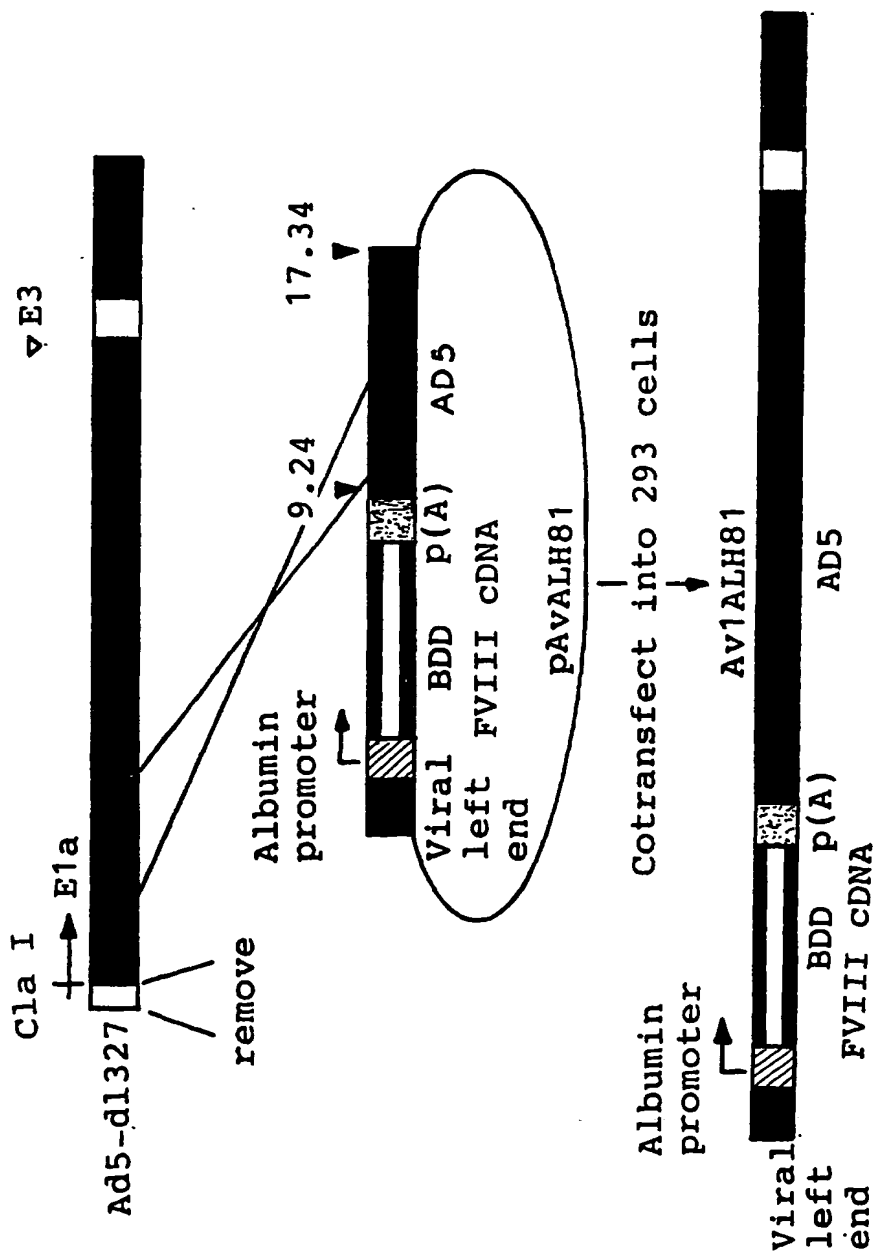
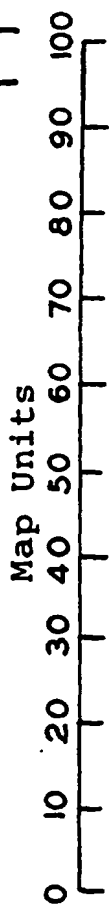


FIG. 37

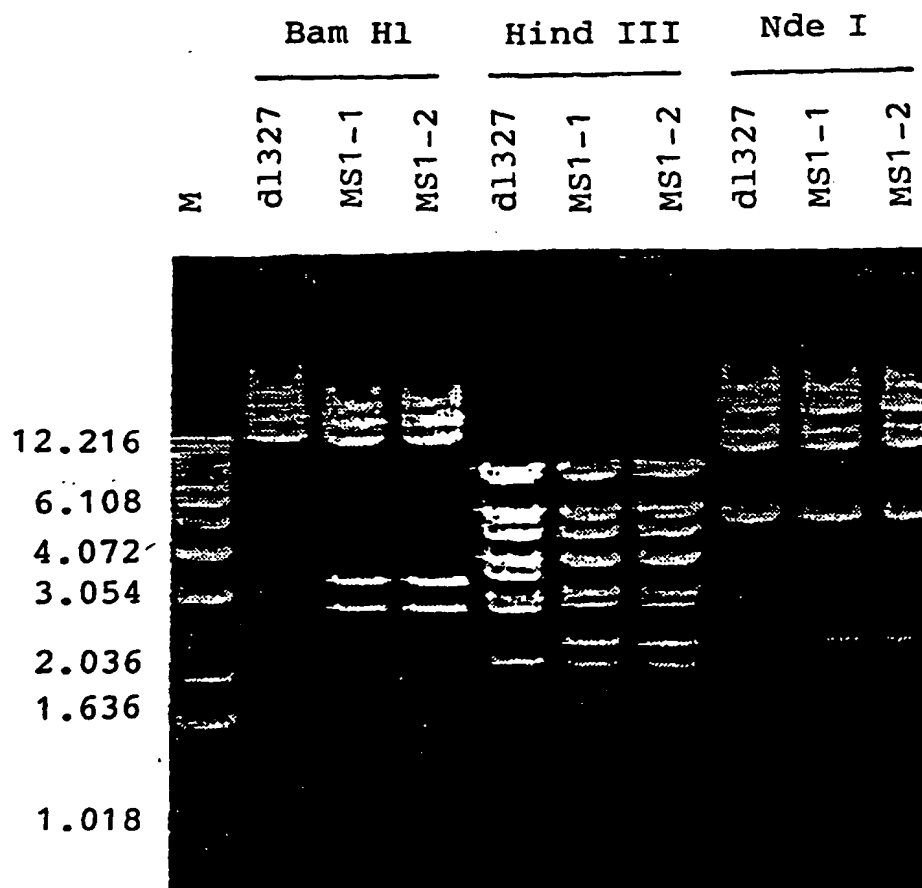
GENERATION OF AV1ALH81





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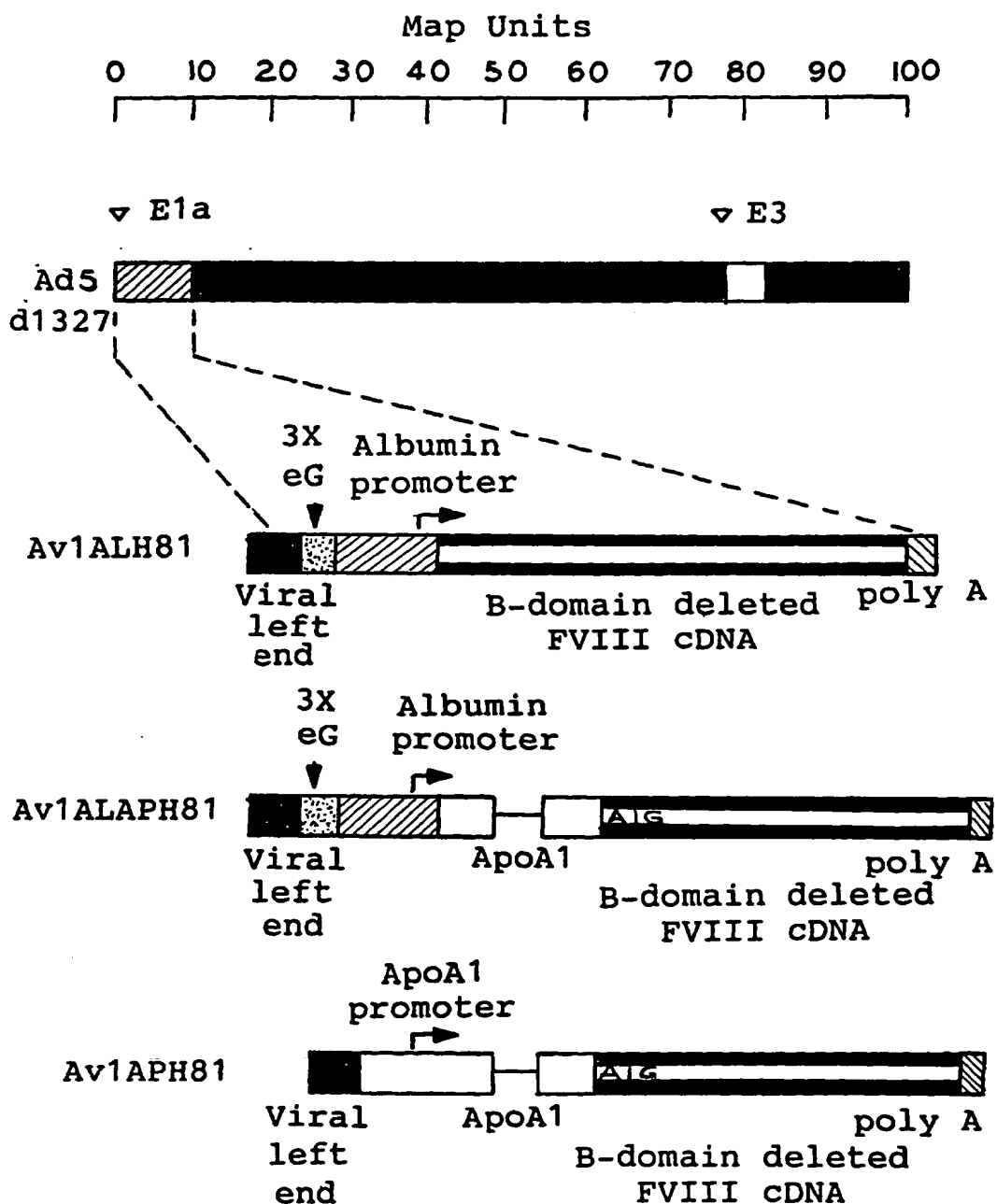
## FIG. 38

RESTRICTION DIGESTION ANALYSIS  
OF Av1ALH81 DNA

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## FIG. 39

## FACTOR VIII ADENOVIRUS VECTORS



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## HUMAN FACTOR VIII-SPECIFIC ELISA

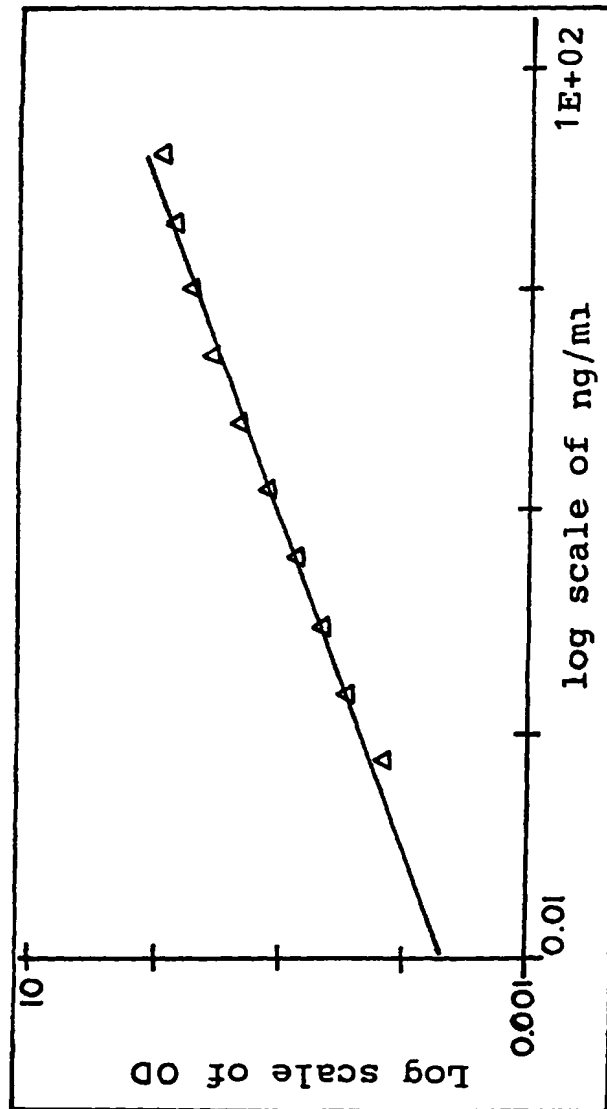
## STANDARD CURVE

FIG. 40

Curve Fit Log-Log

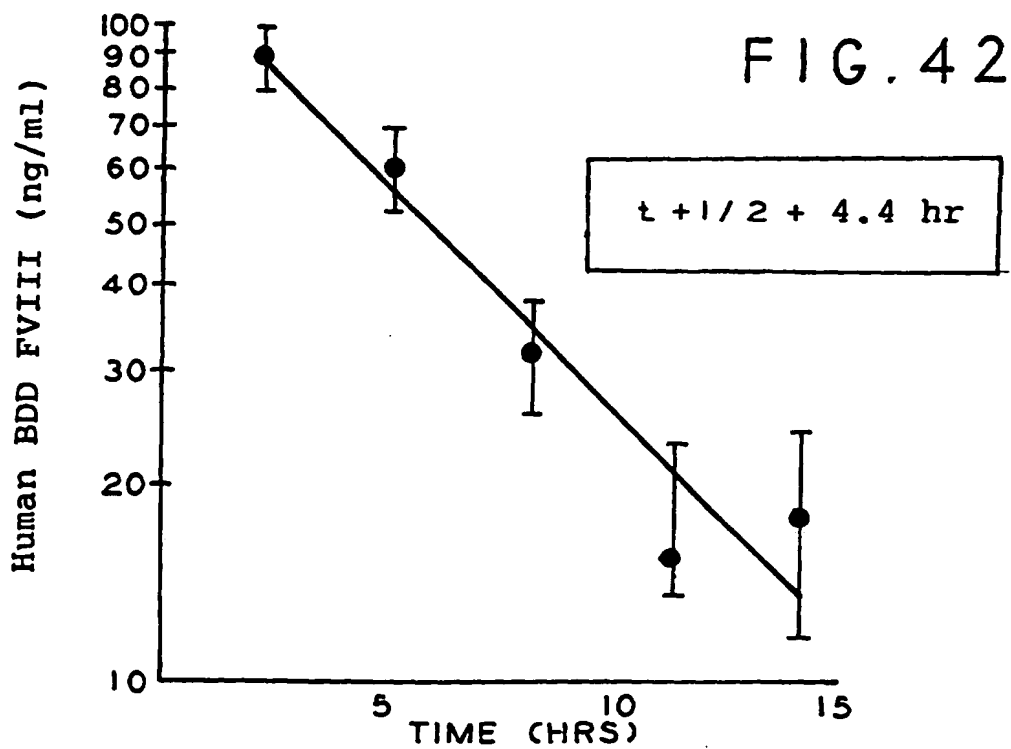
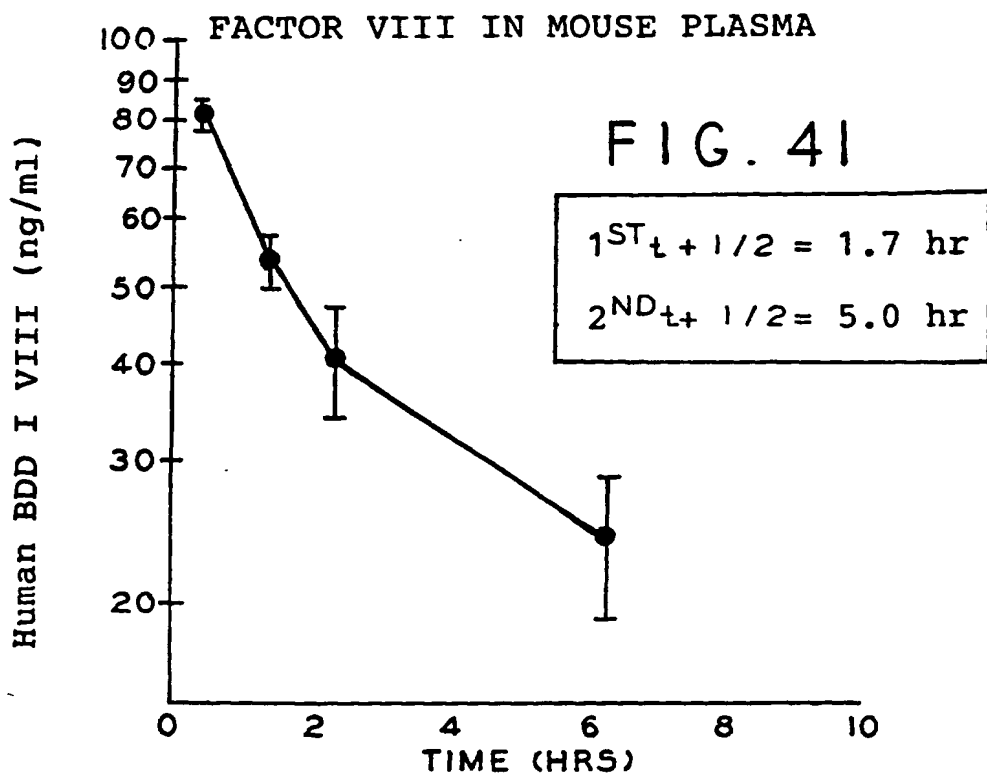
 $\log(y) = A + B * \log(x)$  Corr. Coeff: 0.993

A = -0.964 B = 0.683



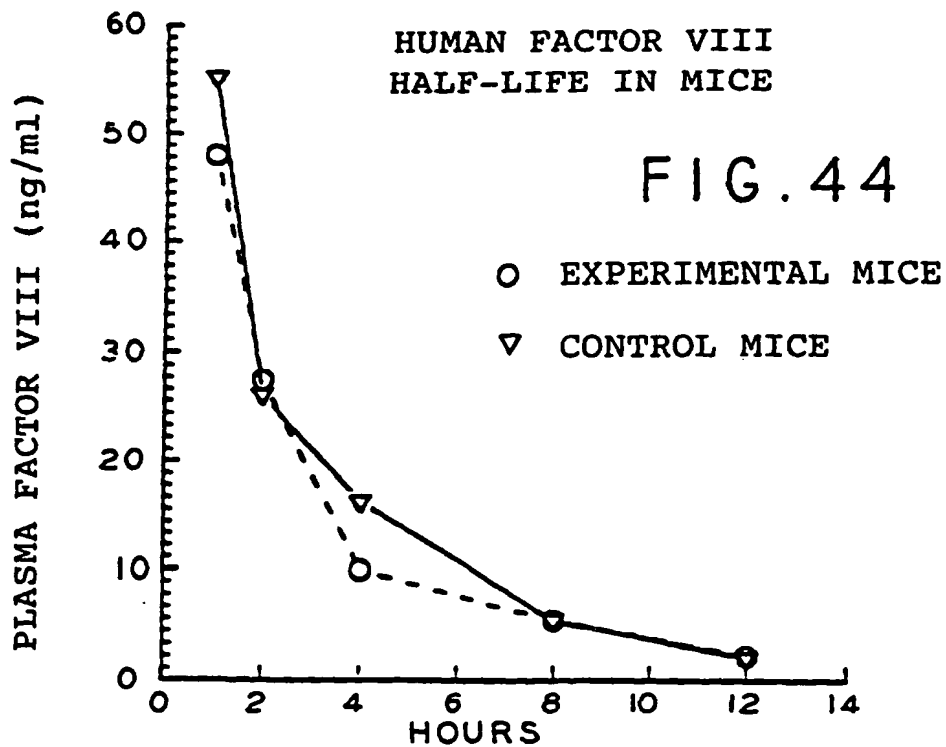
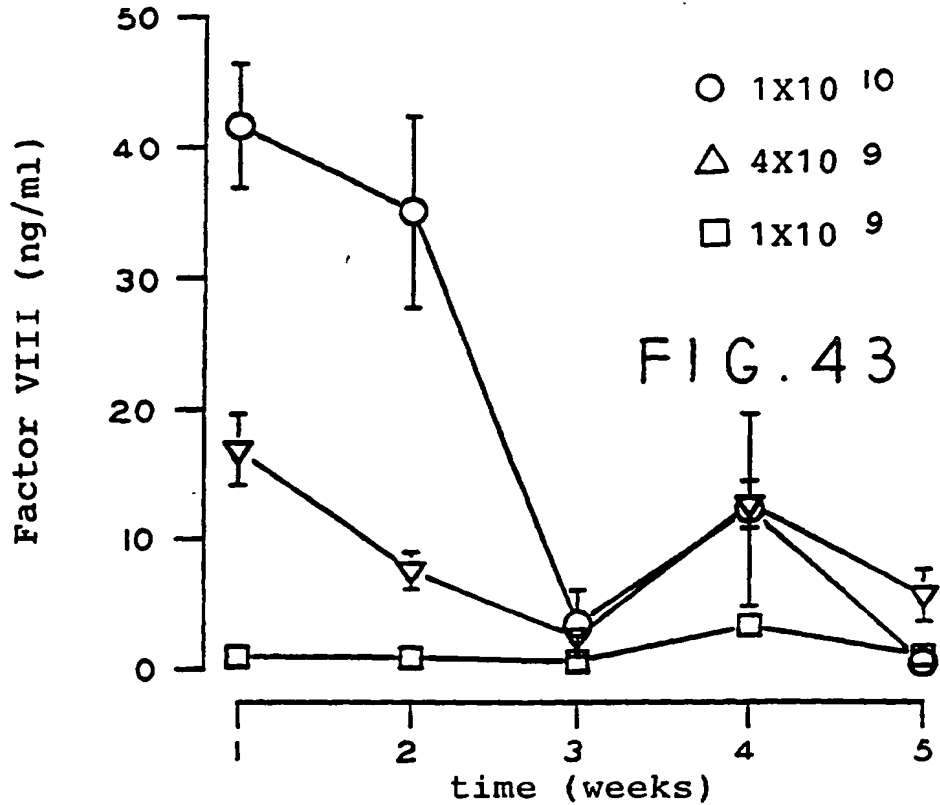
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## HALF-LIFE OF HUMAN B-DOMAIN DELETED

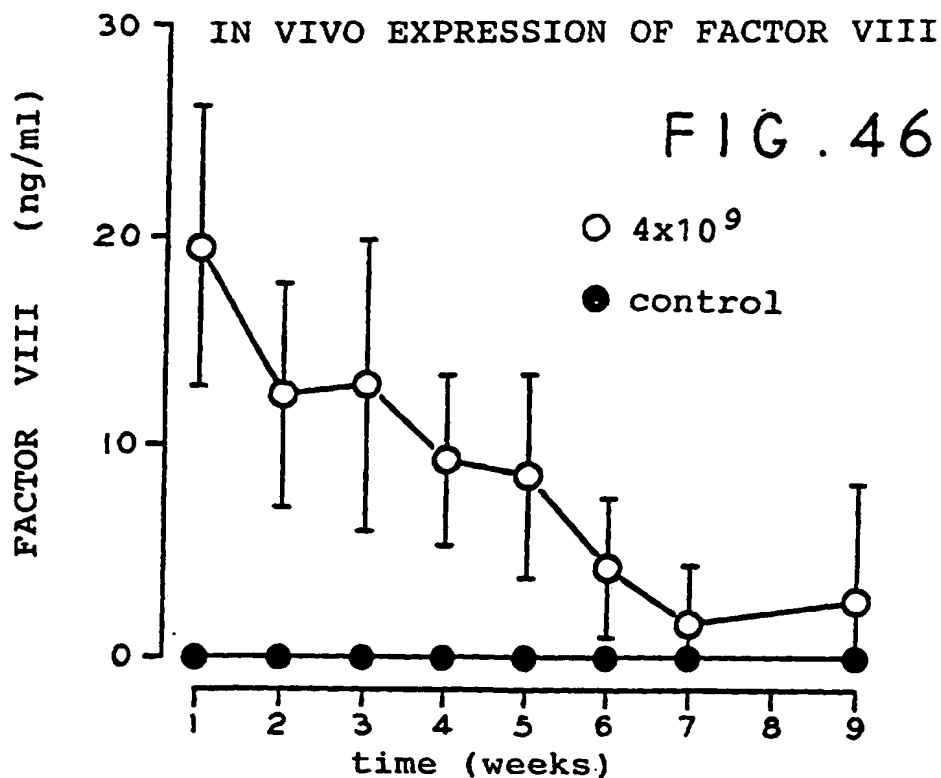
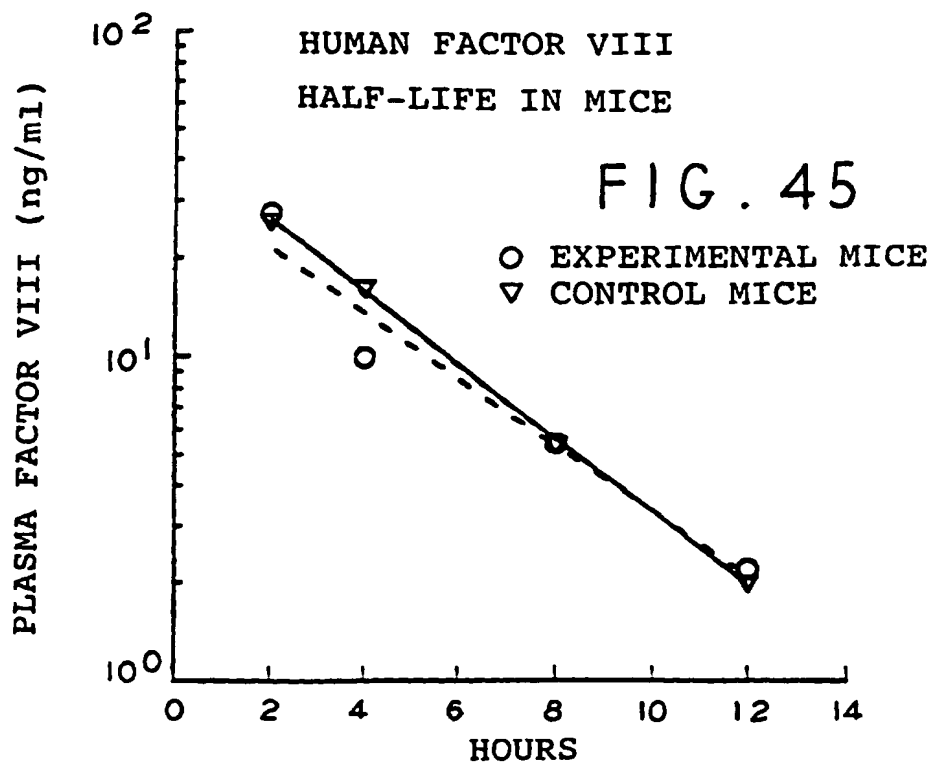


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## IN VIVO EXPRESSION OF FACTOR VIII

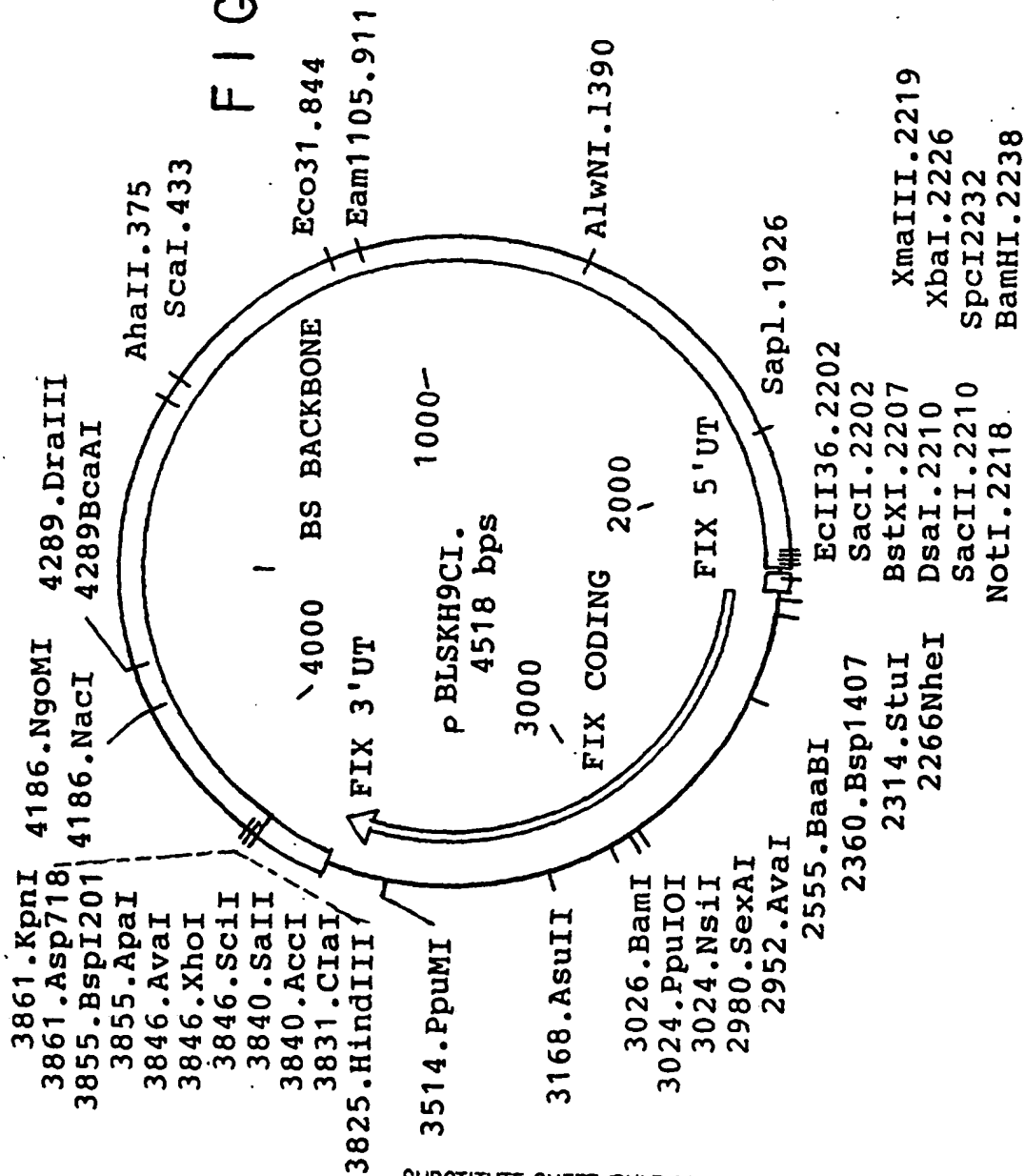


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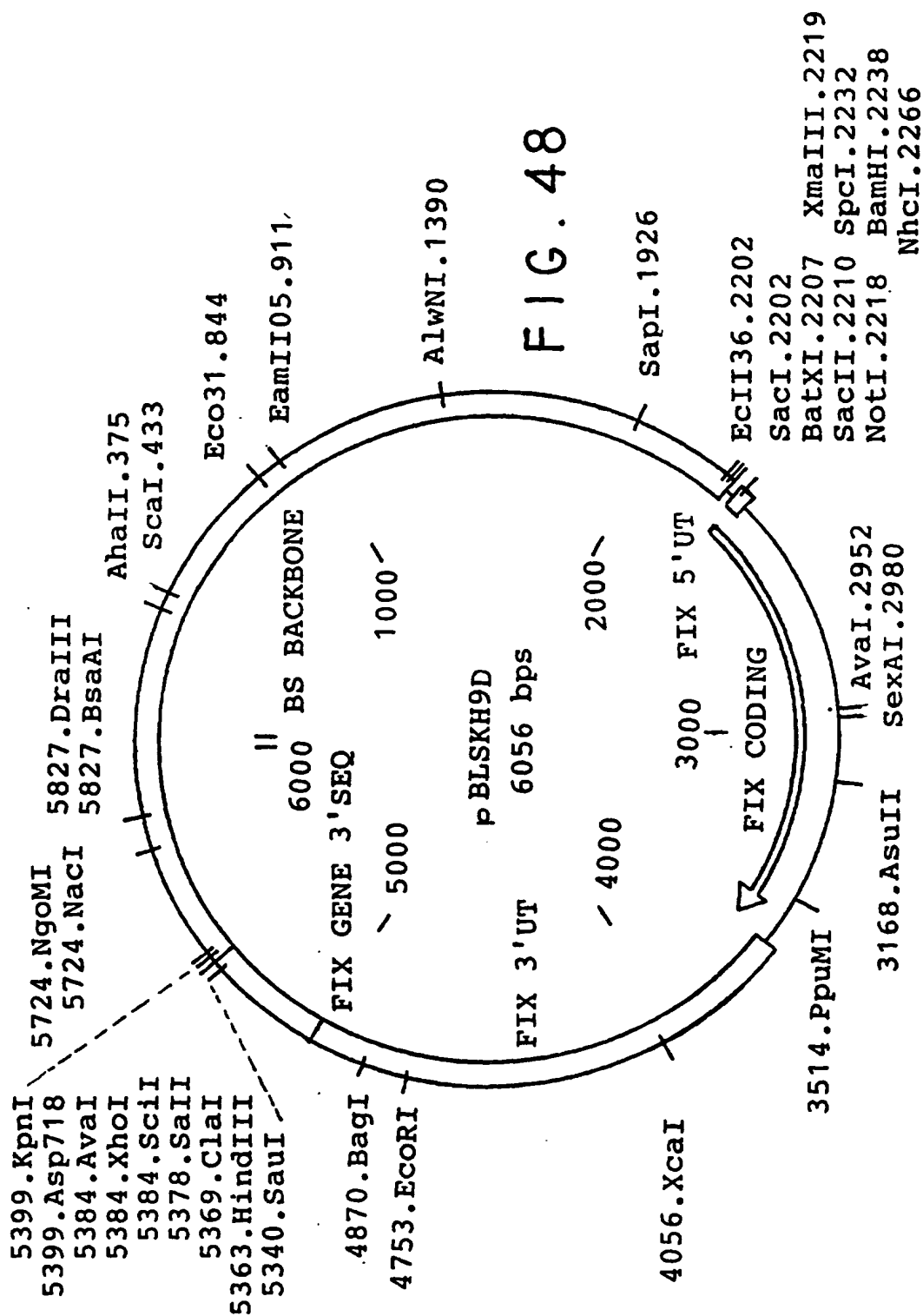
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FIG. 47



SUBSTITUTE SHEET (RULE 26)

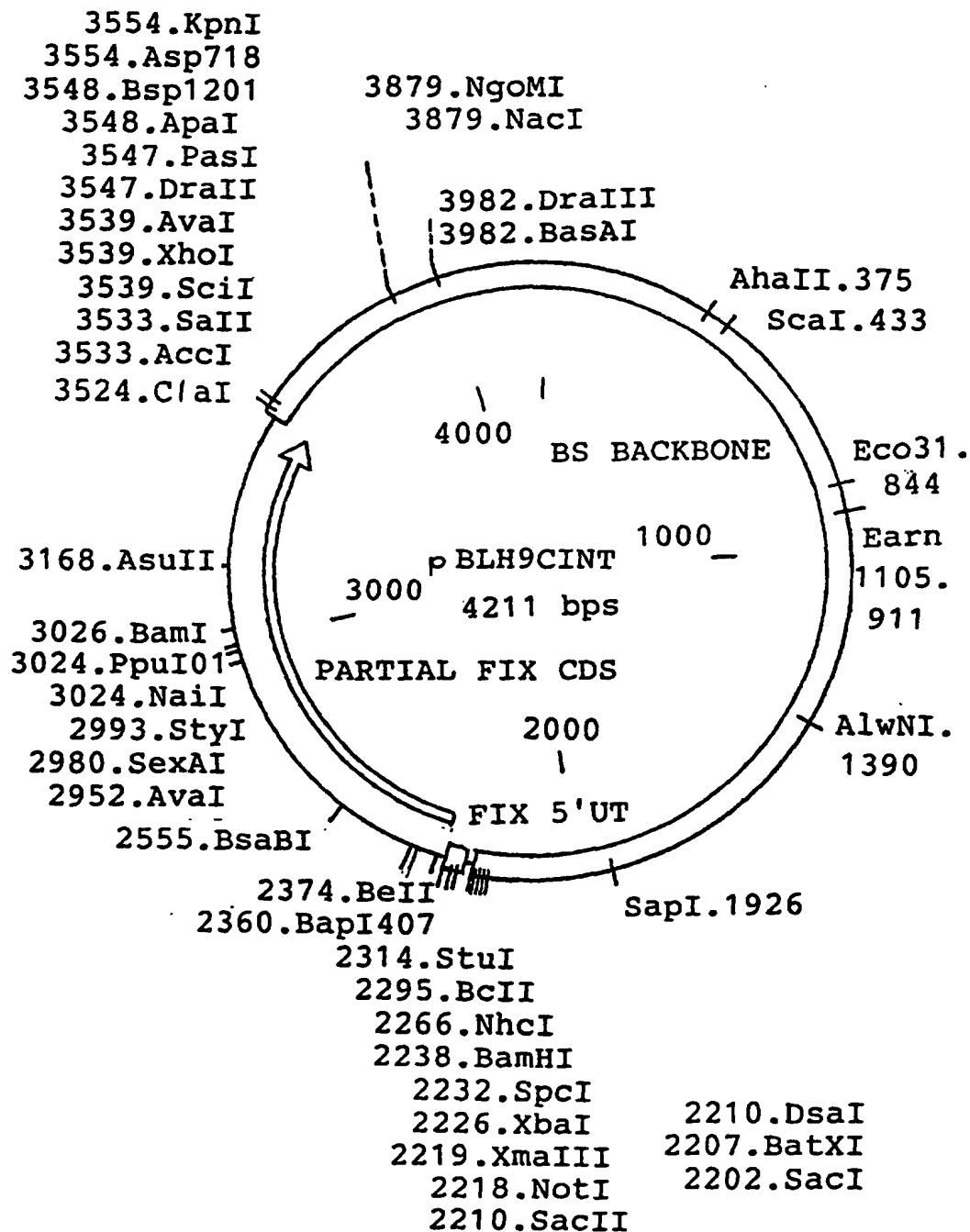
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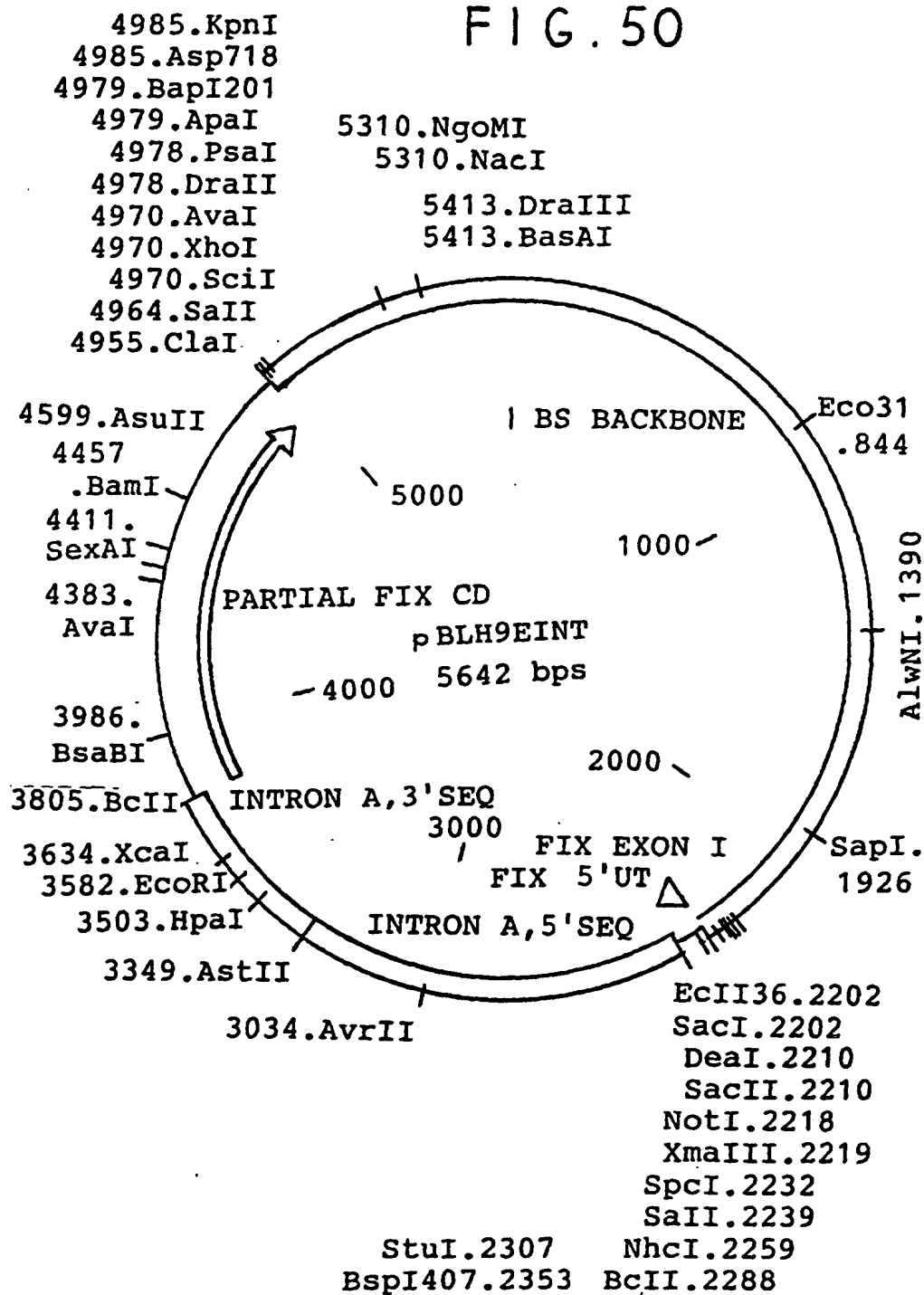
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## FIG. 49



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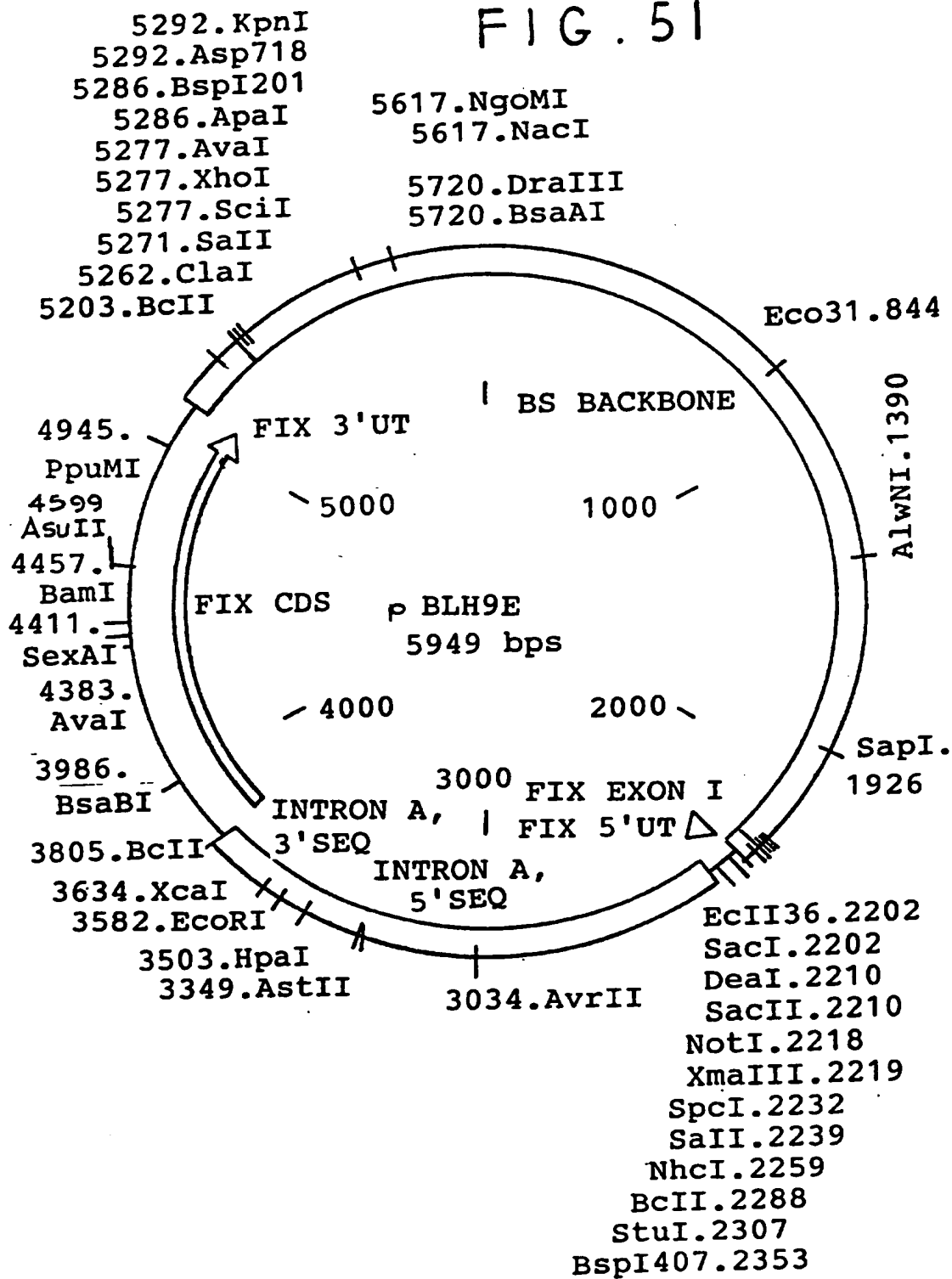
## FIG. 50



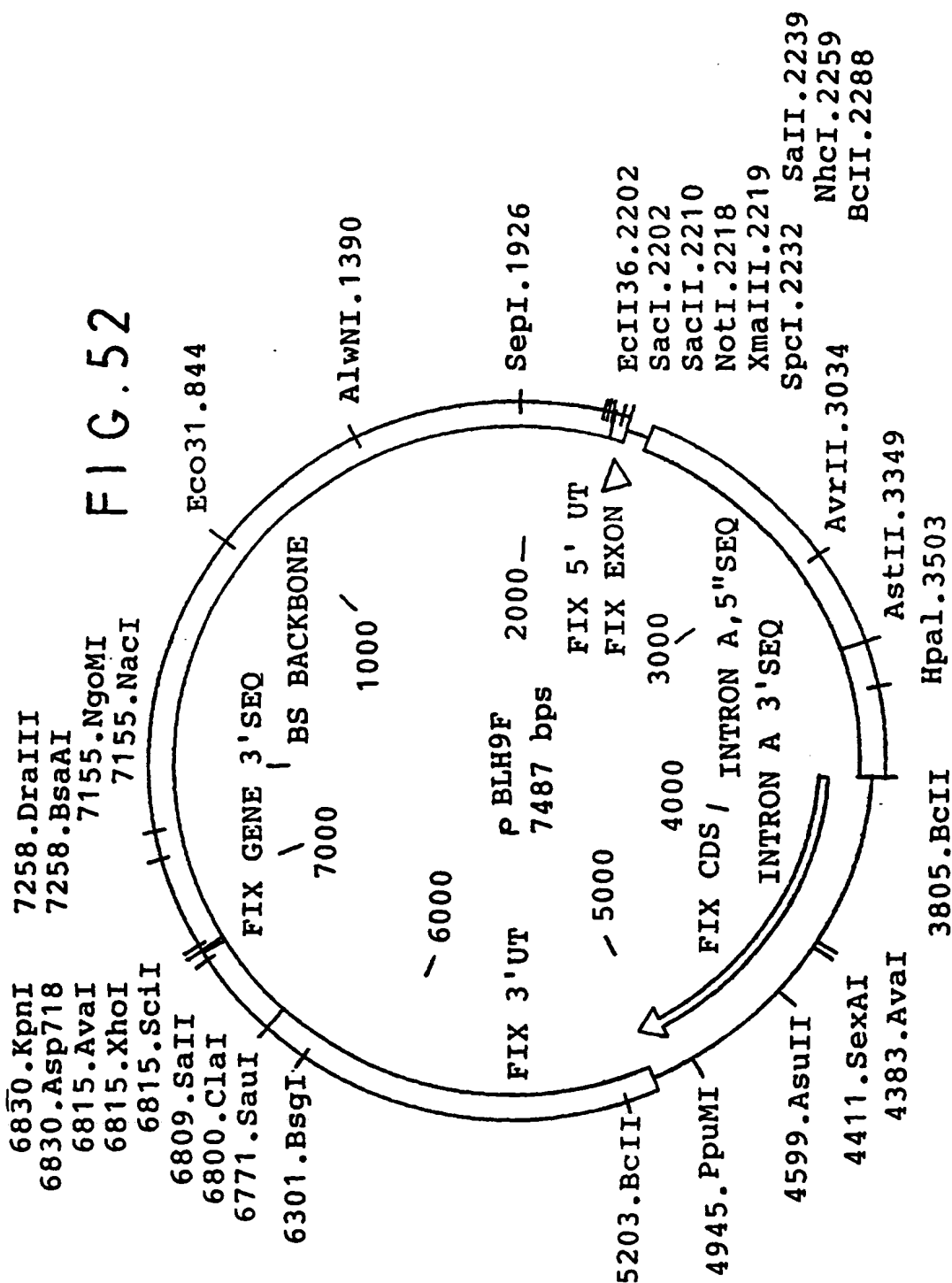
SUBSTITUTE SHEET (RULE 26)

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FIG. 51

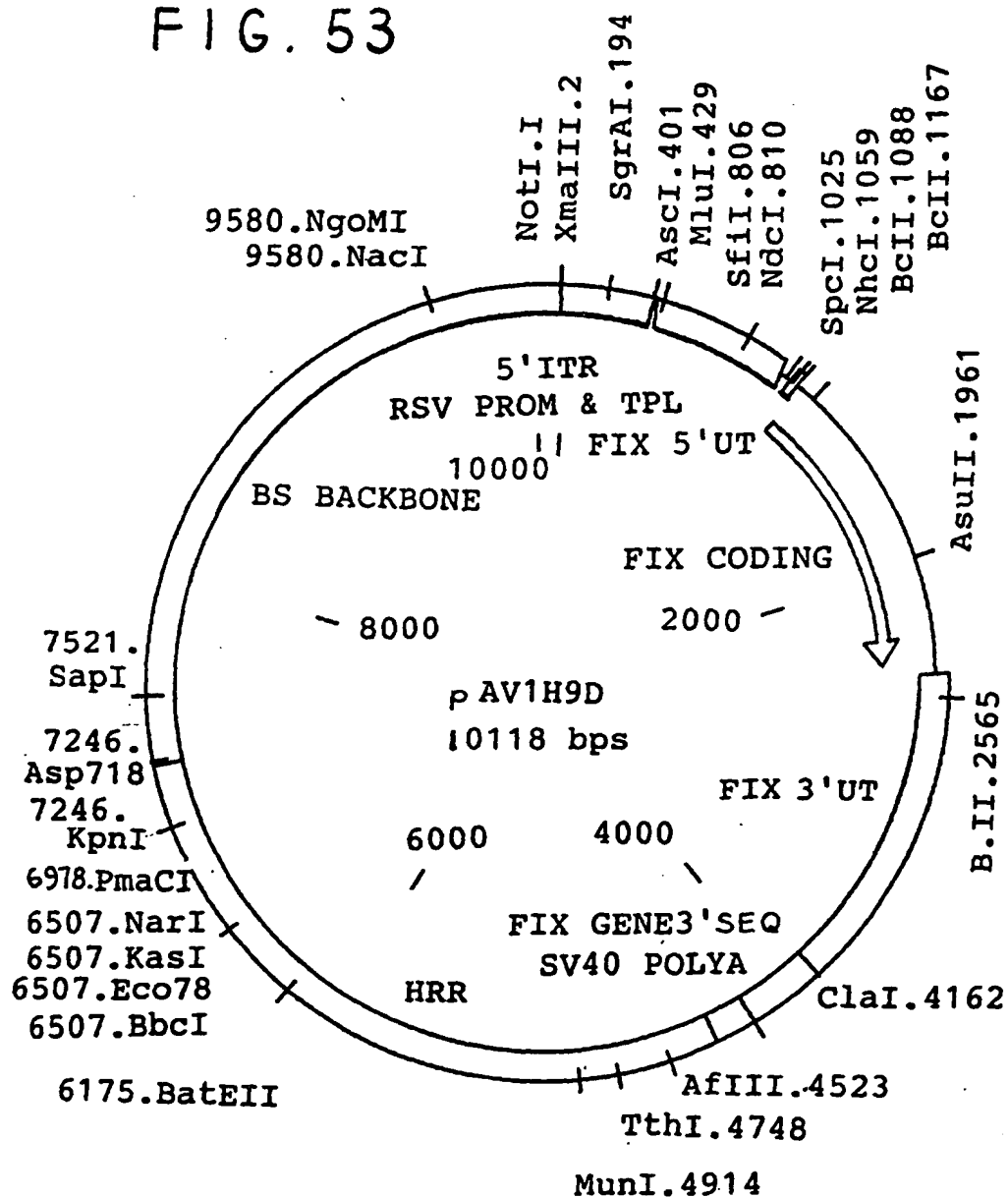


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FIG. 53





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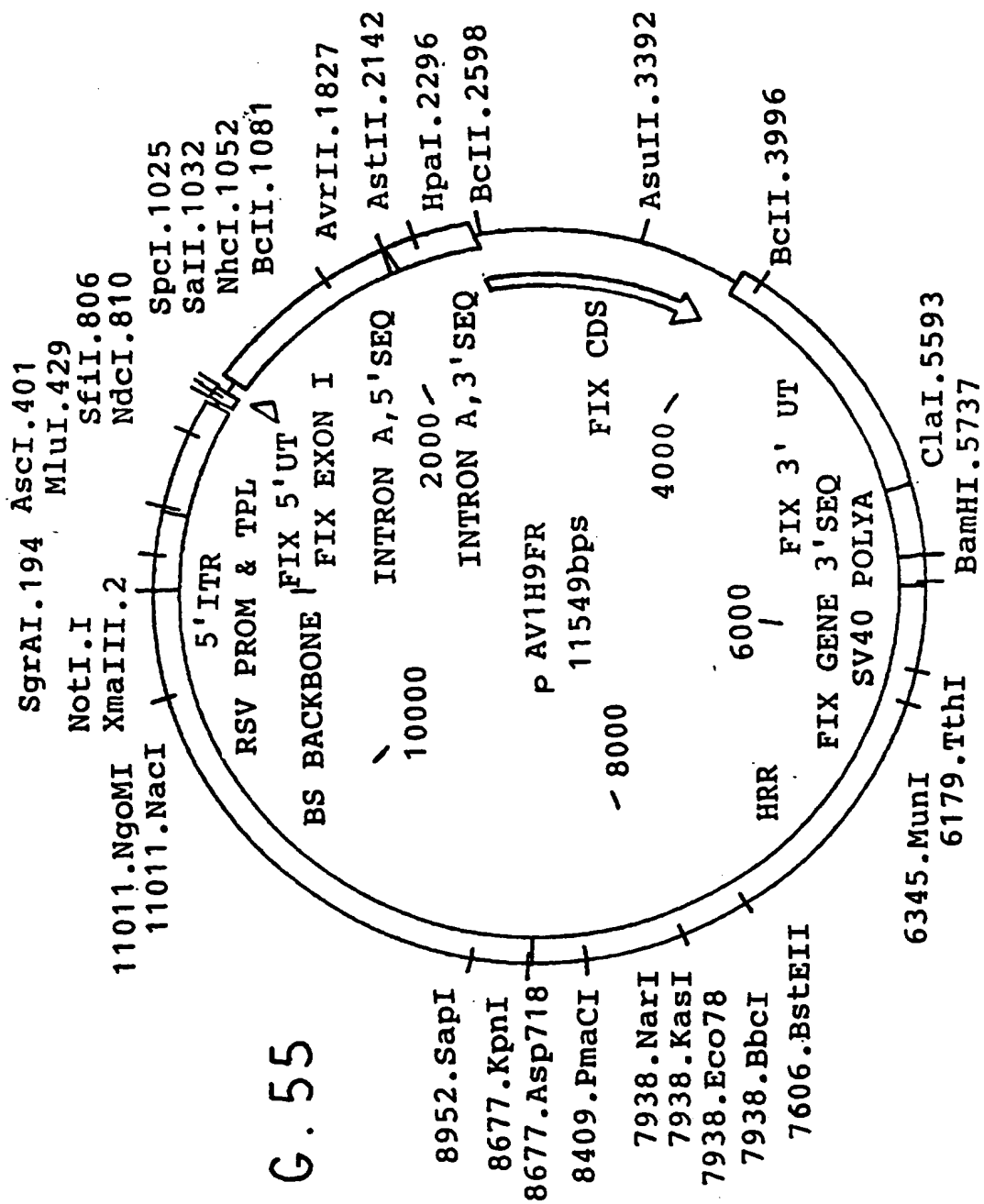
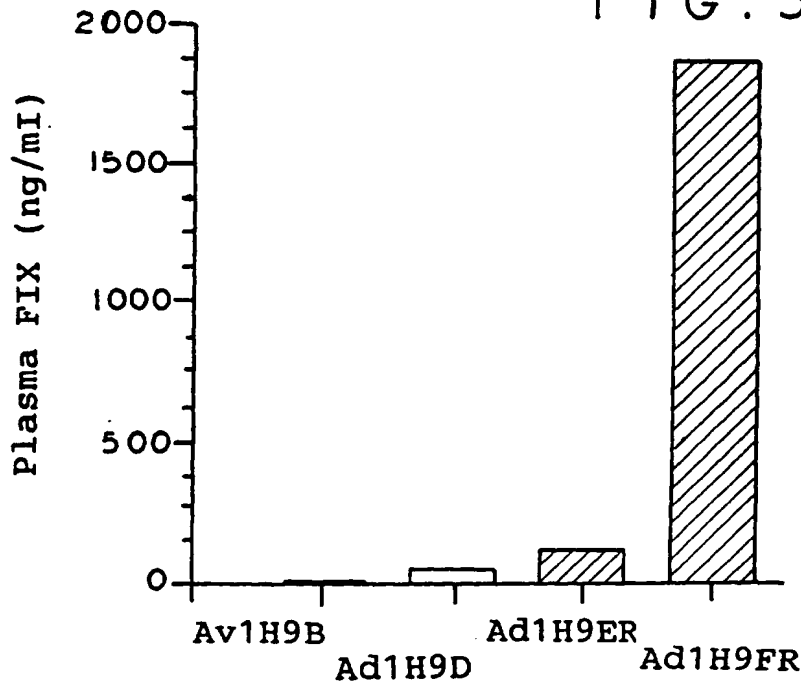


FIG. 55

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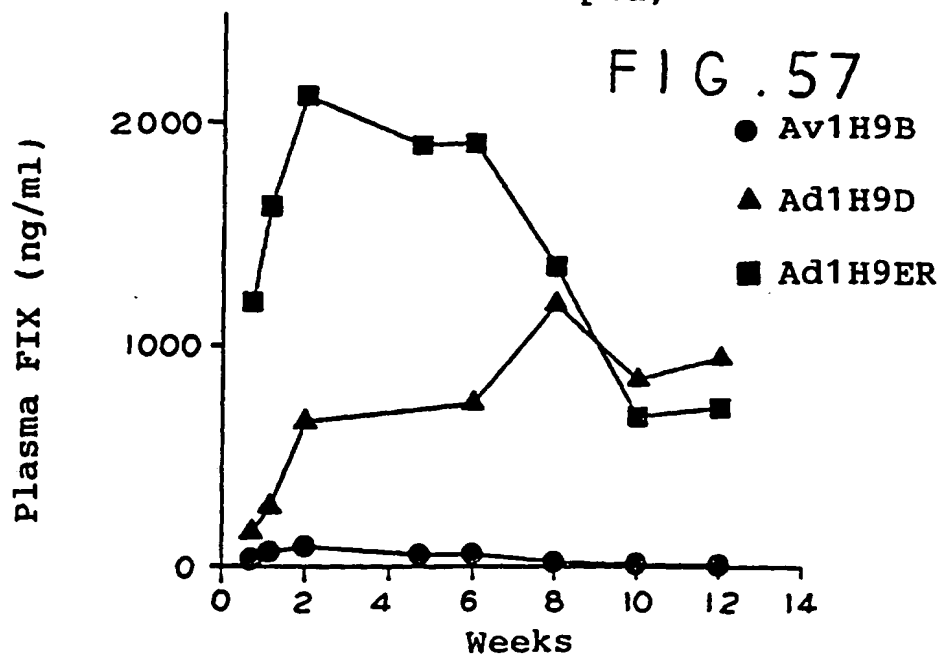
Comparison of Factor IX vectors in Mice  
( $2 \times 10^8$  pfu)

FIG. 56



Comparison of Factor IX Adenovectors in Mice  
( $1 \times 10^9$  pfu)

FIG. 57





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Comparison of Factor IX Adenovectors in Mice

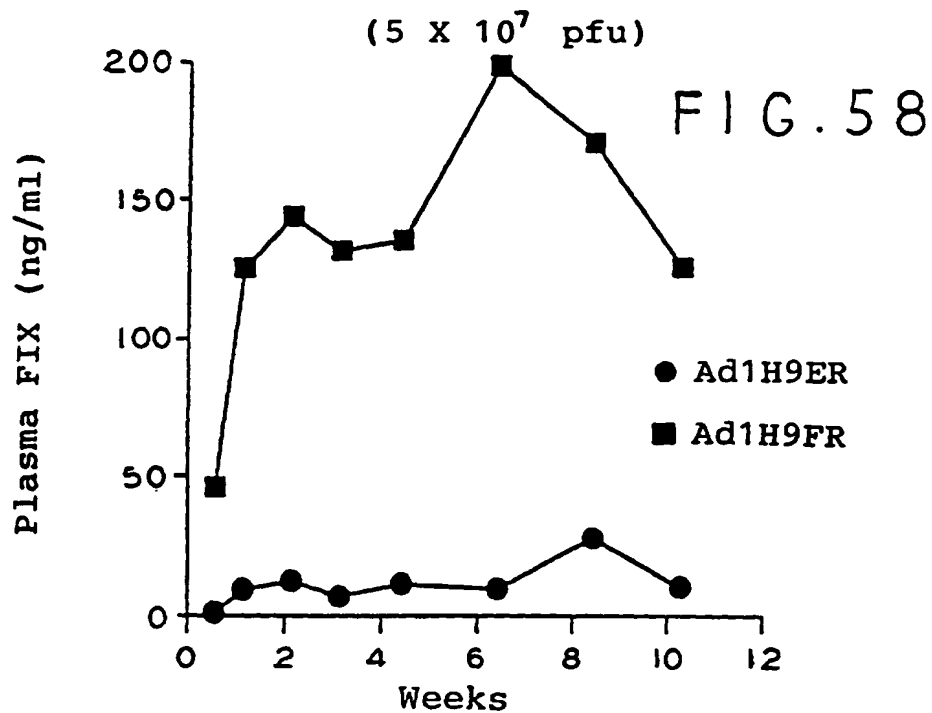
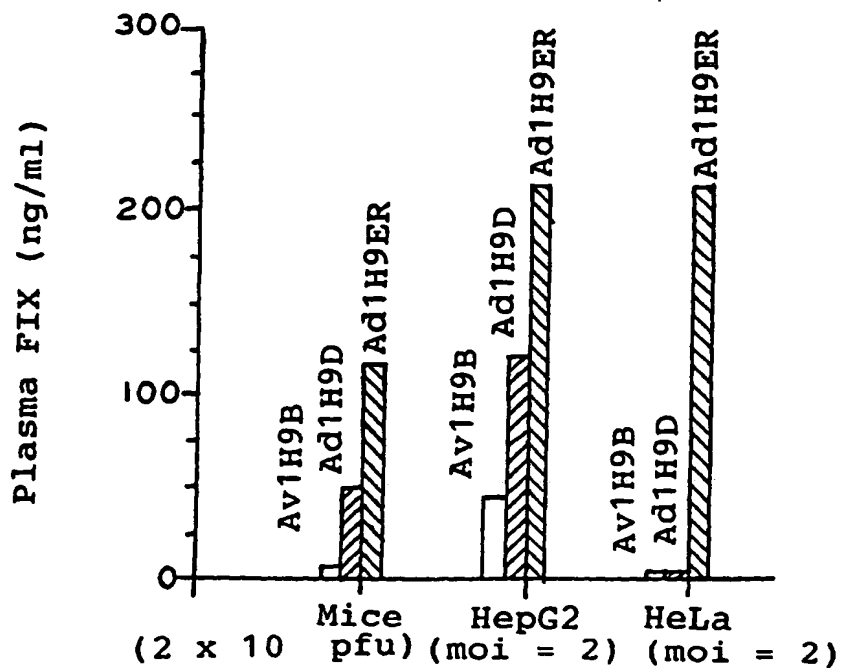


FIG. 59  
Comparison of Factor IX Vectors  
in vivo and in vitro



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